Mechanism and synchronicity of wheat (*Triticum aestivum*) resistance to leaf rust (*Puccinia triticina*) and Russian wheat aphid (*Duiraphis noxia*) SA1

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Declaration

I, the undersigned, declare that this thesis submitted to the University of Fort Hare for the degree of Doctor of Philosophy in Biochemistry in the Faculty of Science and Agriculture is my original work and has never been presented for a degree in any other university, and that all sources of material used for this thesis have been duly acknowledged.

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Dedication

I dedicate this Thesis to my kids; Njom Ryan Njom and Ijang-Mande Zuriel Njom on the basis that the study will be a source of encouragement and also a driving force for them to further their own studies.

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List of abbreviations

AOS	-Allene oxide synthase
Ca^{2+}	-Calcium ion
CSIR	-Council for Scientific and Industrial Research
g/l	-Grams per liter
LOX	-Lipoxygenase
MAMPs	-Membrane-associated molecular patterns
MR	-Moderately resistant
PPO	-Polyphenol oxidase
QTLs	-Quantitative loci
RuBisCO	-Ribulose bisphosphate carboxylase/oxygenase
RWA	-Russian wheat aphid
RWA SA1	-Russian wheat aphid biotype 1
SOD	-Superoxide dismutase
2-DE	-Two dimensional electrophoresis
ABA	-Abscisic acid
ACN	-Acetonitrile
ADP	-Adenosine diphosphate
ATP	-Adenosine triphosphate
ARC-SGI	-Agricultural Research Council-Small Grain Institute
ACO1	-aminocyclopropane-1-carboxylic acid oxidase
$(NH_4)HCO_3$	-Ammonium bicarbonate
APX	-Ascorbate peroxidase
BSA	-Bovine serium albumin
CaEF	-Calcium binding elongation factor
$CaCl_2$	-Calcium chloride
CO_2	-Carbondioxide
CAT	-Catalase
DAMPs	-Damaged-associated molecular patterns
DPT	-Dathiothreitol
DAI	-Days after infection
^oC	-Degrees celcius

DNA	-Deoxyribonucleic acid
ETI	-Effector-triggered immunity
ESI	-Electrospray ionisation
ET	-Ethylene
FAO	-Food and Agricultural Organisation
FA	-Formic acid
BGL1	-β-glucosidase 1
GPX	-Glutathione peroxidase
GST	-Glutathione S-transferase
g	-Gram
HEL	-Hevein-like protein
Hrs	-Hours
H_2O_2	-Hydrogen perioxide
OH^-	-Hydroxyl radicals
HR	-Hypersentivity response
IPG	-Immobilisd p^H gradient
ISR	-Induced systemic resistance
ITs	-Infection types
IEF	-Isoelectric focusing
pI	-Isolectric point
JA	-Jasmonic acid
kDa	-Kilodalton
Lr	-Leaf rust
LCMS	-Liquid chromatography-mass spectrometry
MS	-Mass spectrometry
MALDI	-Matrix-assisted laser desorption ionisation
µg	-Microgram
µL	-Microliters
MS	-Moderately susceptible
M	-Molar

MAS	-Marker-assisted selection
ng	-Nanogram
NADPH	-Nicotinamide adenine dinucleotide phosphate
NO	-Nitric oxide
NL	-Non linear
NBS-LRR	-Nucleotide binding site-leucine rich repeat
PTI	-PAMP-triggered immunity
PAMPs	-Pathogen-associated molecular patterns
PR	-Pathogenesis related
PAL	-Phenyalanine ammonia lyase
PGPR	-Plant growth promoting rhizobecteria
PRPs	-Plasma membrane-localised recognition receptors
PCR	-Polymerase chain reaction
PCD	-Programmed cell death
Pt	-*Puccinia triticina*
ROS	-Reactive oxygen species
R	-Resistant
SA	-Salicylic acid
SDS-PAGE	-Sodium dodecyl sulfate polyacrylamide gel electrophoresis
O_2	-Superoxide
S	-Susceptible
SAR	-Systemic acquired resistance
TRXs	-Thioredoxins
TOF	-Time of flight
UK	-United Kingdom
USA	-United States of America
VSP	-Vegetative storage protein
VOCs	-Volatile organic compounds
V	-Volt

General Abstract

Wheat (*Triticum aestivum* and *T. Durum*) is an extremely important agronomic crop produced worldwide. Wheat consumption has doubled in the last 30 years with approximately 600 million tons consumed per annum. According to the International Maize and Wheat Improvement Center, worldwide wheat demand will increase over 40% by 2020, while land as well as resources available for the production will decrease significantly if the current trend prevails. The wheat industry is challenged with abiotic and biotic stressors that lead to reduction in crop yields. Increase knowledge of wheat’s biochemical constitution and functional biology is of paramount importance to improve wheat so as to meet with this demand. Pesticides and fungicides are being used to control biotic stress imposed by insect pest and fungi pathogens but these chemicals pose a risk to the environment and human health. To this effect, there is re-evaluation of pesticides currently in use by the Environmental Protection Agency, via mandates of the 1996 Food Quality Protection Act and those with higher perceived risks are banned.

Genetic resistance is now a more environmental friendly and effective method of controlling insect pest and rust diseases of wheat than the costly spraying with pesticides and fungicides. Although, resistant cultivars effectively prevent current prevailing pathotypes of leaf rust and biotypes of Russian wheat aphid from attacking wheat, new pathotypes and biotypes of the pathogen/pest may develop and infect resistant cultivars. Therefore, breeders are continually searching for new sources of resistance. Proteomic approaches can be utilised to ascertain target enzymes and proteins from resistant lines that could be utilised to augment the natural tolerance of agronomically favourable varieties of wheat. With this ultimate goal in mind, the aim of this study was to elucidate the mechanism and synchronicity of wheat resistance to leaf rust (*Puccinia triticina*) and Russian wheat aphid (*Duiraphis noxia*) SA1.

To determine the resistance mechanism of the wheat cultivars to leaf rust infection and Russian wheat aphid infestation, a proteomics approach using two-dimensional gel electrophoresis was used in order to determine the effect of RWA SA1 on the wheat cultivars proteome. Differentially expressed proteins that were up or down regulated (appearing or disappearing) were identified using PDQuest^{TM} Basic 2-DE Gel analysis software. Proteins bands of interest were in-gel trypsin digested as per the protocol described in Schevchenko *et al*. (2007) and analysed using a Dionex Ultimate 3000 RSLC system coupled to an AB Sciex 6600 TripleTOF mass spectrometer. Protein pilot v5 using Paragon search engine (AB Sciex)

was used for comparison of the obtained MS/MS spectra with a custom database containing sequences of Puccinia triticina (Uniprot Swissprot), *Triticum aestivum* (Uniprot TrEMBL) and Russian wheat aphid (Uniprot TrEMBL) as well as a list of sequences from common contaminating proteins. Proteins with a threshold of ≥99.9% confidence were reported.

A total of 72 proteins were putatively identified from the 37 protein spots excised originating from either leaf rust or Russian wheat aphid experiments. Sixty-three of these proteins were associated with wheat response to stress imposed by RWA SA1 feeding while 39 were associated with infection by *Puccinia triticina*. Several enzymes involved in the Calvin cycle, electron transport and ATP synthesis were observed to be differentially regulated suggesting greater metabolic requirements in the wheat plants following aphid infestation and leaf rust infection. Proteins directly associated with photosynthesis were also differentially regulated following RWA SA1 infestation and P. triticina race 3SA145 infection including a key enzyme of the Calvin cycle, ribulose bisphosphate carboxylase/oxygenase (RubisCO. These changes observed may reflect the reallocation of metabolites from normal growth processes to defensive functions after induction of plant responses by aphid feeding as well as pathogen infection. Some of the differentially accumulated proteins were found to be involved in ATP synthesis. The increased energy demand by the infected or infested plants was to cope with the impeding stress. Also, a few of the differentially regulated proteins were found to be related to stress. Most of these proteins were expressed early in the resistant cultivar but only occurred much later in the moderately resistant cultivar. These results suggest that RWA SA1 infestation and leaf rust infection of wheat may result in an increase photosynthesis, photorespiration, ATP synthesis and production of stress related proteins to cope with the stress.

The effect of leaf rust infection on wheat plant colonization with RWA was evaluated. Two wheat cultivars, SST 347 (resistant to both leaf rust race 3SA145 and Russian wheat aphid biotype SA1) and SST 356 (susceptible to both 3SA145 and RWA SA1) were grown to the 2-3 leaf stage. Treatments consisted of untreated control (wheat seedling infested with aphids only) and test (wheat seedlings inoculated with urediniospores of *Puccinia triticina* race 3SA145 suspended in Soltrol-170® and later infested with aphids at day 3, 5, 7 and 9 post infection). Aphid population on each plant was determined by counting the total number of aphids on each leaf with the aid of a hand lens for 21 days post infestation. Disease response in inoculated seedlings was scored using the 0 to 4 infection type scale. Results obtained showed infection types; 1+ and 3++ for SST 347 and SST 356 respectively. Few aphids chose to colonise previously infected plants at the beginning as aphid numbers on the preinfected SST 347 only reached >10 after day 16 whereas on preinfected SST 356 reached >10 after day 13-15. We therefore concluded that preinfection with leaf rust delayed aphid colonization of both the resistant SST 347 and susceptible SST 356 cultivars to the same extent. Therefore prior infection of wheat plants under laboratory conditions appears to induce resistance to Russian wheat aphids (antixenosis) in both resistant and susceptible cultivars. However, this resistance was later overcome with doubling of aphid populations occurring at a higher rate on the susceptible cultivar as compared to the resistant cultivar. This was the first study to show that infection of plants with rust primed resistance to insect herbivores.

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CHAPTER ONE

LITERATURE REVIEW

1.1 General introduction

Wheat is a cereal plant of the genus *Triticum* and is one of the first domesticated food crops in the world. Its ability to self-pollinate greatly facilitated the selection of many distinct domesticated varieties (Peter, 2009). According to FOAStat (2015), 720 million tons of wheat was produced globally in 2014 making it the third most-produced cereal after maize (1,016 million tons) and rice (745 million tons). Although wheat has served as food to millions of people all over the world, it is the basic staple food in Europe, West Asia and North Africa (Curtis, 2002). Today, wheat is grown on more land area than any other commercial crop and continues to be the most important food grain for humans (FAO, 2007). The European Union ranks first in world wheat production, followed by China which is also the World's largest importer, with more than 10 million tons imported annually on average since 1980 (FAO, 2014).

On the African continent, the cultivation of wheat dates back to the early history of mankind (Murdock, 1960). However, the early wheat was different to today's wheat, which has been bred to grow faster, be resistant to abiotic and biotic stresses and provide a higher yield per hectare (Gupta *et al.*, 2010). The most cultivated varieties of wheat are the common (bread) wheat (*T. aestivum*) and the durum wheat (*T. durum*) which both account for about 95% and 5% of world wheat production respectively (Curtis, 2010; Peng *et al.*, 2011).

Wheat is classified into spring or winter wheat, traditionally referring to the season during which the crop is grown. *Triticum* spp originated from the Levant region of the Near East and Ethiopian Highlands and is cultivated throughout the world (Tanno and Willcox, 2006; Dixon *et al.*, 2009). It is the most traded cereal in the world out performing all other crops combined and serves as the leading source of vegetable protein for humans (Curtis, 2002). Wheat grain remains an important source of protein and starch, both of which provide energy for the human body. It is also an important source of dietary fibre, antioxidants and other phytochemicals which are particularly enriched in whole grain products (Shewry, 2009; JO'Connor, 2012). All these factors are of importance to public health as it can prevent cardiovascular diseases, diabetes and colon cancer (NDA, 2011; Navas-Loper *et al*., 2014). However, wheat products are responsible for a number of adverse conditions in humans including intolerances notably coeliac disease and allergies (respiratory and food) (Shewry, 2009).

In South Africa, wheat is the second most important grain cultivated after maize. Although the Western Cape, Northern Cape and Free State provinces account for the majority of the country's output, small amounts are grown in the Northern Cape, North West, Mpumalanga, Limpopo, KwaZulu-Natal, Eastern Cape and Gauteng (DAFF, 2013; van de Vyfer and Nordier, 2013). Most of the wheat cultivated is the bread wheat used mainly for the production of bread and other products such as biscuits and breakfast cereals. However, in some areas of the country, small amounts of durum wheat (*T. durum*) are cultivated and used for the production of macaroni, spaghetti and other pasta products (DAFF, 2013).

Wheat yields are often affected by several factors including biotic and abiotic stresses. Biotic stresses include insect infestation and infection by microorganisms, while abiotic stresses include extreme temperatures, CO_2 concentrations, drought, salt (salinity) and heavy metals (Vinour and Altman, 2005; Pretorius *et al*., 2007). Infection with leaf (brown) rust (*Puccinia triticina*) is one of the most important grain limiting factors throughout the small grain production areas of South Africa (Pretorius *et al*., 2007; Terefe *et al*., 2014). Although the disease occurs regularly in most wheat growing regions, it is more frequent in the winter rainfall regions of the Western Cape and areas where wheat is grown under irrigation (Pretorius *et al.*, 2007; Terefere *et al*., 2009). Wheat yield decreases have also been attributed to infestation by the phloem feeding insect, the Russian wheat aphid (RWA) which has been referred to as a notorious pest that reduces wheat yields significantly (Botha *et al*., 2014; Mohase and Taiwe, 2015).

Breeding resistant cultivars remain the most effective, economic and environmental friendly method for the control of wheat diseases and insect pest (Du Toit, 1988; Pretorius *et al*., 2007). However, breeding for a new resistant cultivar requires extremely long timeframes; it could take more than fifteen years between the initial cross made for a cultivar and the first release of commercial seed (Le Maitre and Botes, 2013). Knowledge of pathotype (race) composition of the current and future rust population is required for breeding of resistant cultivars and has been acquired through population genetics studies of wheat rusts and predictions made to future pathogen population (Botha and Venter, 2000). Several of these resistance genes have been discovered and mapped on the chromosome but the mechanism of resistance conferred by the genes is not fully established (Vanzetti *et al*., 2011). Formerly, the genetic basis of resistance was thought to follow the gene-for-gene hypothesis (Flor, 1971) but recently it is more complex.

Over the years, there has been a remarkable increase in the World's population and it is expected to reach 9.2 billion by 2050 (http://factsanddetails.com).The agricultural sector feels the pressure more than any other sector and will need to feed approximately 2.3 billion more people than at present. One of the ways to sustain this ever growing population is by providing consistent, high quality and high yield wheat for human consumption (FAO, 2011). The transfer of resistant genes to adapted cultivars plays an important role to achieve this goal. Molecular markers (MAS) have been developed and are used to select plants with important traits to reduce timeframe for breeding (Botha and Venter, 2000). Little however is known about the proteins that are involved in plant resistance mechanisms (Rampitsch *et al*., 2006). This study was therefore under taken to determine which wheat proteins are differentially regulated in either wheat/aphid or wheat/leaf rust interactions which could be involved in resistant mechanisms and be used as markers to assist breeders select for disease/insect pest resistant wheat cultivars. Two dimensional gel electrophoresis (2-DE) and Mass spectrometry (MS) was employed to investigate some of the changes in the wheat proteome of two wheat cultivars (SST 356 and SST 347) when stressed with RWA SA1 or infected with leaf rust race 3SA145.

1.2 Wheat leaf (brown) rust

1.2.1 Introduction

Leaf (brown) rust caused by *Puccinia triticina* is an important and widely distributed disease of bread wheat (*Triticum aestivum* L.) and durum wheat (*T. durum*) worldwide (Bockus *et al*., 2010; Kolmer, 2013). In South Africa, leaf rust occurs annually almost everywhere where wheat is cultivated in the country. It is most prevalent on autumn-sown spring wheat grown in the winter rainfall regions of the Western Cape and severe but less frequent epidemics have been recorded on winter wheat in the Free State Province and on irrigated spring wheat in other areas (Pretorius *et al*., 1987; Terefe *et al*., 2009). The distribution and severity of the disease is influenced by free water on the surface of the host plant, ambient temperatures, density of inoculum, and susceptibility of cultivars, fungicide application and climatic conditions during the growing season (Pretorius *et al*., 1987; Waggoner and Aylor, 2000; Pretorius and Bender, 2010). Although leaf rust infections are less damaging than that of stem or stripe (yellow) rust, its annual, frequent and widespread occurrence results in higher total annual losses (Huerta-Espino *et al*., 2011). In South Africa, devastating epidemics are rare, although higher annual yield losses due to leaf rust infection have been documented (Pretorius *et al*., 2007). Annual leaf rust surveys in South Africa have shown diversity in pathogenicity and the ability of the pathogen to overcome leaf rust resistance in wheat; this has been the primary reason for cultivar replacement (Pretorius *et al*., 1990; Terefe *et al*., 2009). Fungicides have been effective in the control of leaf rust but their high cost and negative effect on the environment has made plant pathologists seek alternative sources for control (Marais and Botes, 2003; Marais *et al*., 2009). The use of resistant wheat cultivars offers the most effective and ecologically sustainable method of leaf rust control and genetic incorporation of resistance genes into adapted germplasm remains the major goal of most wheat breeding programs (Marias *et al*., 2009). Currently, more than 71 leaf rust resistance (*Lr*) genes are known although many of them are no longer used in agriculture as they have been overcome by virulent races (McInstosh *et al*., 2012). Resistance is either conferred by a single resistance gene (seedling or race specific resistance) or by multiple genes or quantitative trait loci (QTLs) (adult plant, race non-specific or slow rusting resistance) (Kou and Wang, 2010; Prins *et al*., 2011).

The leaf rust fungus is classified into numerous physiologic races (pathovars) like most cereal rusts. More than 150 races are known to exist and have become virulent on cultivars that were previously resistant (Terefe *et al*., 2009). Severe epidemics have been reported in many wheat production areas with the appearance of virulent pathotypes (Pretorius *et al*., 1990). The diversity and the ability of *P. triticina* to overcome leaf rust resistance requires a constant monitoring of changes in the pathogen population and the search for new and effective resistance sources (Kloppers and Pretorius, 1995; Pretorius and Bender, 2010). In South Africa, regular annual monitoring of the leaf rust pathogen only began in the 1980's (Pretorius and Le Roux, 1988; Terefe *et al*., 2009), forty three years after the pioneer survey done by Verwoerd in 1937 (Verwoerd, 1937). Information obtained from the surveys is always important to breeders for the development of new resistant cultivars.

1.2.2 Physiological races (pathotypes) and distribution

Over the past 30 years, annual rust surveys have been undertaken in South Africa by the Agricultural Research Council-Small Grain Institute (ARC-SGI), the country's National Monitory Program. Results obtained have significantly contributed to the progress achieved thus far in breeding resistant and high yielding wheat cultivars (Terefe and Pretorius, 2010). Because of the importance of leaf rust in global wheat production, surveys to determine diversity and distribution of *P. triticina* populations were also instituted in other countries. Surveys were started in the USA in 1926 (Johnston *et al*., 1968), in 1920 in Australia (Waterhouse, 1952) and in 1931 in Canada (Johnson, 1956). In South Africa, the first leaf rust survey was conducted in 1937 by Verwoerd (Verwoerd, 1937) and based on the physiological characteristics of the pathogen, five races were reported. Since then the pathogenicity of wheat leaf rust remained largely unknown until the institution of regular surveys in the 1980's (Pretorius *et al*., 1987; Pretorius and Le Roux, 1988). Leaf rust races were characterised by examining their infection types (ITs) on various wheat lines or cultivars carrying particular *Lr* genes. A unified standard system for naming of leaf rust races was also created (Pretorius *et al*., 1987). Races are named by alpha-numerical code consisting of three digits with the first being the rust type; 2SA is stem rust and 3SA is leaf rust with the following digits being a sequential record number for the pathotype. The code is also accompanied by avirulence/virulence formula (Le Roux and Rijikenberg, 1987).

A total of 15 *P. triticina* races were reported between 1983 and 1988 (Pretorius *et al*., 1990; Pretorius *et al*., 1987; Pretorius and Le Roux, 1988) and presently 18 races have been reported in South Africa (Visser *et al*., 2012). Nine pathotypes designated as 3SA120,

3SA121, 3SA122, 3SA123, 3SA124, 3SA125, 3SA126, 3SA127 and 3SA128 were identified in the 1985 survey (Pretorius *et al*., 1987). The isolates were characterised by the ITs on the following cultivars with respective *Lr* genes; RL6003 (*Lr*1), RL6016 (*Lr*2a), RL6019 (*Lr*2b), RL6002 (*Lr*3a), RL6042 (*Lr*3bg), RL6007 (*Lr*3ka), RL6004 (*Lr*10), RL6053 (*Lr*11), RL6013 (*Lr*14a), RL6052 (*Lr*15), RL6005 (*Lr*16), RL6008 (*Lr*17), Thew (*Lr*20), Agent (*Lr*24) and RL6049 (*Lr*30). Between 1986 and 1987 leaf rust commonly occurred, but was most severe on winter wheat in the Orange Free State (Pretorius and Le Roux, 1988). A total of seven races were identified in both years but pathotypes 3SA134 (1986) with avirulence/virulence formula *Lr*3a, 3bg,3ka, 10, 11, 16, 20, 24, 26, 30/1, 2a, 2b, 2c, 14a, 15, 17 and 3SA140 (1987) with *Lr*3a, 3bg, 3ka, 11, 16, 20, 30/1, 2a, 2b, 2c, 10, 14a, 15, 17, 24, 26) were characterised for the first time.

During the 1988 survey, 11 pathotypes were found with races 3SA126 and 3SA133 being the most frequently isolated and four races (3SA137, 3SA141, 3SA142 and 3SA143) occurring for the first time (Pretorius *et al*., 1990). A general trend observed in this survey was recurrent epidemics of leaf rust, as most of the wheat cultivars used were susceptible. Although *P. triticina* was widespread on wheat, levels of severity were low and extensive yield losses were not observed. A new leaf rust race, with unusual virulence characteristics was reported in the 2005 annual survey. This race designated 3SA144 which was the first to be reported with virulence to the leaf rust resistance gene *Lr*32 was collected from triticale in the Western Cape. However, this *Lr*32 resistance gene was not being used in local cultivars (Pretorius and Bender, 2010). It was revealed that race 3SA144 clustered closely with race 3SA132, although it preferred triticale, the original host in which it was isolated. Pretorius and Bender (2010) suggested that race 3SA144 could have originated from one of the races 3SA132, 3SA134, 3SA137 and 3SA140. In the 2007 annual survey, Terefe *et al*. (2009) characterised five pathotypes from a total of 80 leaf rust samples collected. The pathotypes observed had virulence patterns similar to those previously reported. However, they noted that race 3SA133 (76.8%), found in samples from all the localities, occurred more frequently, and followed by pathotype 3SA126 (11.0%). Other pathotypes detected were 3SA140 (7.3%), 3SA132 (3.7%) and 3SA137 (1.2%). During the 2009 survey, seven leaf rust races were characterised, with six having virulence patterns similar to those observed in previous surveys; one was a new leaf rust race (Pretorius and Bender, 2010). This race, designated as 3SA145 was collected from the Western and Eastern Cape Provinces and was different from other known races due to its combined virulence for the adult-plant resistance genes *Lr*12, *Lr*13 and *Lr*37 (Terefe *et al*., 2011; Visser *et al*., 2012). In South Africa, where the alternate host that allows for the completion of the life cycle is absent, new races develop from existing populations by mutation, somatic recombination or could enter an area as foreign introduction (Terefe *et al*., 2014). In such an area when susceptible wheat cultivars are grown, these new races will establish themselves and eventually dominate the population.

1.2.3 Wheat leaf rust resistant genes and mechanisms of resistance

Wheat researchers world-wide have conducted several genetic studies on leaf rust resistance in wheat with the first done by Mains *et al.* (1926) who reported the presence of a resistant gene in two wheat cultivars Malakof and Webster that conferred resistance to leaf rust. These resistant genes were later designated as *Lr*1 and *Lr*2 respectively (Ausemus *et al*., 1946). In another study by Soliman *et al*. (1964), *Lr* genes were mapped by identifying the chromosomes that carried leaf rust resistance genes *Lr*1, *Lr*3 and *Lr*11. Later, allelic variation in Lr genes was demonstrated by Dyck and Samborski (1968) when they determined the presence of three alleles at the *Lr*2 locus. Recently, leaf rust resistance genes designated *Lr*1 to *Lr*60 have been described and genes characterised in common wheat (*T. aestivum*), durum wheat (*T.durum*) and many wild wheat species (McIntosh *et al*., 2007). In the hexaploid wheat, Lr genes are widely distributed across the genome on almost every one of the 42 chromosome arms. Four allelic series have been described on different chromosome arms. “The *Lr*2a, *Lr*2b, *Lr*2c as well as *Lr*22a and *Lr*22b genes have been mapped to a locus on the chromosome arm 2-DS” (McIntosh and Baker, 1968; Rowland and Kerber, 1974). “The *Lr*3a, *Lr*3ka and *Lr*3g genes are located on a locus on chromosome arm 6BL” (Haggag and Dyck, 1973), “*Lr*17a and *Lr*17b are at a locus on chromosome arm 2AS” (Dyck and Kerber, 1977). “Other genes such as *Lr*14a and *Lr*14b are strongly linked on chromosome 7BL and are considered as alleles for all practical purposes” (Dyck and Samborski, 1970).

Although Lr genes confer resistance to most leaf rust races, the precise mechanism of action has not been fully understood. Most of these genes have effective resistance to specific races of *P. triticina* with some effective to multiple races. At the interface between fungal haustoria and host cells in the epidermal and mesophyll layers, race-specific resistance is manifested by a hypersensitive response (HR) characterised by rapid cell death (Bolton *et al*., 2008). However, different resistance genes form characteristic resistance phenotype or infection types. It has been observed that while the resistance response of wheat lines with the *Lr*3 gene are characterised by clearly defined hypersensitive flecks, those with *Lr*2a show very light flecks which might not be visible (Kolmer *et al*., 2007). Other race-specific resistance responses including those with *Lr*3ka, *Lr*3bg and *Lr*11genes exhibit small uredinia surrounded by chlorosis while lines with *Lr*16 have small uredinia surrounded by necrosis expressed at a later stage in the infection and colonisation process (Mishira *et al*., 2005; Kolmer *et al*., 2007).

Race-specific Lr genes are effective in seedling plants and remain effective in adult plant stage although that formed by genes, such as *Lr*12, *Lr*13 and *Lr*22a, are best expressed in the adult plants. It is important to note that some genes that confer race-specific resistance have been successfully cloned and they include *Lr*1 and *Lr*10, originally from common wheat and *Lr*21, original from *T. tauschii* (Feuillet *et al*., 2003; McIntosh *et al*., 2012). "These genes form HR response to isolates of *P. triticina* that bear corresponding avirulence genes, and encode proteins with nucleotide binding site-leucine rich repeat (NBS-LRR) regions; characteristic of most plants disease resistance genes. All Lr genes do not form race-specific resistance however, *Lr*34 was the first to be described as a modifier of adult plant resistance in the cultivar Frontana" (Dyck *et al*., 1966). Thereafter, the gene has been found in several wheat lines from around the world (Dyck and Samborski, 1982) and was later mapped to chromosome arm 7DS (Dyck, 1987). The *Lr*34 gene forms a resistance response with no HR but exhibits fewer and smaller uredinia with no chlorosis or necrosis on flag leaves of adult plants. Spielmeyer *et al*. (2008) showed that the *Lr*34 gene forms the same resistance response to all isolates of *P. triticina* that were tested (i.e. non race-specific resistance), and was associated with non-HR resistance to stripe rust and powdery mildew. Another gene known to confer adult plant resistance was *Lr*46, originally found in the cultivar Pavon 76. Cultivars with this gene present with fewer and smaller uredinia, but varying amounts of chlorosis in adult plants (Singh *et al*., 1998).

1.2.4 Identification of wheat leaf rust resistant genes

Gene pyramiding is an approach used to stack multiple genes into a single genotype (Joshi and Nayak, 2010). Before gene pyramiding is practiced, it is important to identify effective and genetically different sources of resistance (Joshi anmd Nayak, 2010; Vanzetti *et al*., 2011). Gene postulation is often the method used in breeding companies to determine the presence of probable race-specific seedling resistance genes (*Lr* gene) in a host cultivar (Oelker and Kolmer, 2004; Mebrate *et al*., 2008; Terefe *et al*., 2011). In gene postulation, a host cultivar is tested against a collection of *P. triticina* isolates carrying different avirulence/virulence gene combinations (pathotypes) on the basis of phenotypic expression in the form of infection types (ITs) (Terefe *et al*., 2011). Infection types produced on a series of differential genotypes carrying individual *Lr* resistant genes forms the basis for comparing the ITs of wheat cultivars with unidentified genes for leaf rust resistance (Terefe *et al*., 2011; Vanzetti *et al*., 2011). The limitation of gene postulation is that, it is carried out using rust isolates with known virulence and the procedure is extremely time consuming, requires enough space and is labour intensive and cannot be employed if different fungal isolates are not available (Schnurbusch *et al*., 2004; Vanzetti *et al*., 2011).

Presently, the presence of Lr gene is being determined by testing host cultivars with molecular markers (a piece of DNA which forms part of the wheat chromosome and is linked closely to the gene of interest) linked to resistance genes. This method overcomes some of the challenges associated with traditional gene postulation such as gene interactions and plant stage of gene expression (Langudah *et al*., 2009; Sun *et al*., 2009; Samsampour *et al*., 2010). Molecular markers have been developed recently for several leaf rust resistance genes and this has facilitated the assessment of wheat genotypes with important traits (Vanzetti *et al*., 2011). The molecular markers most closely linked to Lr genes have been developed using PCR based techniques and the majority of these can be applied easily in wheat breeding programmes. In resistance breeding, molecular markers serve two purposes; (i) to monitor the incorporation of designated resistance genes or QTLs into elite wheat genotypes (MAS, marker-assisted selection) and (ii) to identify resistance genes in varieties where genetic background is unknown (gene detection) (Prins *et al*., 2011; Imbaby *et al*., 2014). With the advent of MAS, the long period (approximately 25 years) of developing new crop varieties can now be shortened by the use of molecular markers, to select useful traits. In countries such as Australia, Canada, China, India, Pakistan, South Africa and USA where wheat is a major crop, a great deal of information on postulated leaf rust resistance genes is available (Sing *et al*., 2001; Oelke and Kolmer, 2004; Pathan and Park, 2006; Prins *et al*., 2011).

1.2.5 Breeding for leaf rust resistance

Over the past 30 years, development of adapted wheat cultivars for South African conditions has been the main goal of research initiatives. New resistant cultivars are released or introduced only if they have better agronomical as well as better flour quality characteristics than the cultivars planted commercially in a specific area (ARC-SGI, 2015). Wheat producers continuously strive to improve the wheat yield and quality by selecting the best cultivars for commercial production (ARC-SGI, 2015). Four wheat independent provincial breeding programmes existed before they were merged into one National programme as suggested by Dr. Norman Bolaug (the father of the "Green Revolution" and 1970 Nobel Peace prize winner) (Smit *et al*., 2013). This led to the birth of the Small Grain Centre in 1976, presently known as the ARC-Small Grain Institute in Bethlehem which is the country's National breeding programme (Van Niekerk, 2001). Two private wheat breeding institutions also exist and they are Sensako, established in the mid-1960s and recently became autonomous after functioning as part of Monsanto since 1999 and Pannar that began breeding in the early 1990's (Van Niekerk, 2001). Breeding resistant cultivars is a long process and requires an accurate picture of the current and future pathogen population (Le Maitre and Botes, 2013).

Several resistance genes, in a pre-breeding program created at the Stellenbosch University in 2002, have been transferred from wild species to bread wheat to increase the pool of rust resistance sources (Marais *et al*., 2009). In several instances promising stem rust and/or stripe rust resistance genes were co-transferred with leaf rust resistance genes. Two genes with temporal designation *Lr*S8 and *Yr*S8 from *Triticum dicoccoides* accession 479 obtained from Israel in 1983, were transferred to common wheat (*Triticum aestivum)* (Marais *et al*. 2003). Both genes confer resistance to known South African pathotypes of *P. triticina* and *P. striiformis* respectively. The leaf rust and stripe rust resistance genes *Lr*54 and *Yr*37 have also been transferred to common wheat from *Aegilop kotschyi* (Marais *et al*., 2005). These genes, that occurred on the additional chromosome of the 8078 line, were translocated to chromosome 2DS of common wheat. Other genes transferred to common wheat include the wheat leaf rust resistance gene *Lr*59 derived from *Aegilops peregrine*; *Lr*62 and *Yr*42 from *Aegilops neglecta* Req. ex Bertol (Marais *et al*., 2009).

1.3 Plant defence mechanisms

1.3.1 First line (passive) defence

Plants in both natural and cultivated populations defend themselves from a wide range of invaders but this has not always protected them (Dangl and Jones, 2001). For pathogens to gain access to nutrients from their host, they must first bridge the natural barriers presented by healthy plants. The first constitutive defence barriers that prevent pathogen entry are the cuticle of epidermal cells and suberized cell walls which contain cutin and suberin respectively (Freeman and Beattie, 2008). These molecules are hydrophobic fatty acid like polymers that resist biological degradation except by specialised enzymes. In order for haustorium-forming pathogens to cause disease, they must penetrate the cell wall to establish haustorial feeding structures. However, *Puccinia triticina* does not penetrate the epidermis directly but rather does so through the stomatal opening (Bolton *et al*., 2008). Therefore, rapid closure of the stomata will prevent the rust fungus from gaining access to the host plant (Melotto *et al*., 2008). Papillae (in which secondary antimicrobial metabolites accumulate) deposition at the site of pathogen detection serves as a physical barrier to limit access of pathogens to the protoplast (Clay *et al*., 2009). Successful halting of the invading pathogen by the cell wall-mediated defences at an early stage eliminates the requirement for more costly defence responses such as the hypersensitive reaction (HR) cell death (Morel and Dangl, 1997). Cuticle and cell wall thickness influence resistance to certain pathogens by reducing the ability of the pathogen to enter via the thick and tough cell walls (Freeman and Beattie, 2008). Furthermore, thick cuticles physically prevent the eruption and release of fungal spores, likewise a waxy cuticle prevents the formation of moisture films on leaf surfaces inhibiting fungal spore germination (Figure1.1).

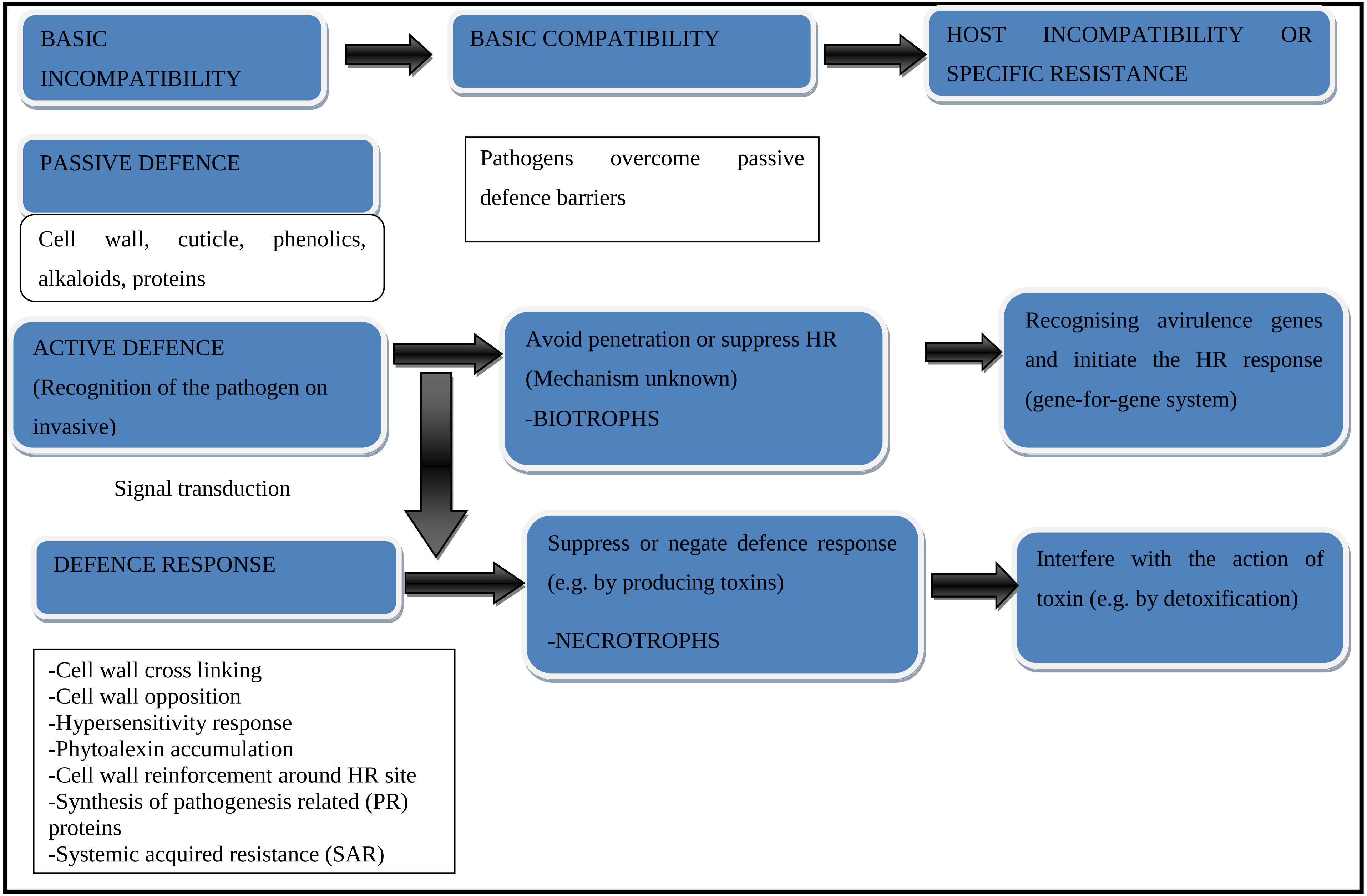


Figure 1.1: Schematic representation detailing mechanisms underlying resistance and disease development in plant pathogen-interaction (Adapted from Johal *et al*., 1995).

1.3.2 Biochemical defences

Two major categories of plant chemicals exist; primary (sugars, proteins, amino acids and nucleic acids) and secondary metabolites (terpenoids, phenols and alkaloids). The primary metabolites are the first substances produced that are important in plant growth and development while the secondary are involved in plant defence (Wittson and Gershenzon, 2002). Plants can also synthesise chemicals such as anti-microbial phytoalexins and saponins that are directly detrimental to pathogens (Bolton *et al.*, 2010). Proteins such as protease inhibitors and lytic enzymes such as chitinases and glucanases are also produced by the plant upon attack (Ryan and Jagendorf, 1995). Defensive chemicals are toxic to the plant; costly in biosynthesis, have ecological consequences and are produced only after initial damage (Gershenzon, 1994; Purninglon, 2000). However, this strategy could be risky because initial attack might be too rapid or too severe for the damage-induced defences to be deployed effectively. Consequently, the plants that are likely to suffer frequent or serious damage may invest mainly in constitutive defences whereas those that are rarely attacked rely on induced defences (Mckey, 1979).

1.3.3 Induced or active defence

The exposure of plants to various pathogens or environmental stress can lead to the activation of inducible defence mechanisms (Ton *et al.*, 2009). Induced defence response is dependent on the recognition of the pathogen by the plant which is specific and can distinguish between different races of the pathogen. The effectiveness of the resistance response is dependent on the rapid recognition of the pathogen-encoded effector protein (avr) by the host resistance (R) gene, a phenomenon known as effector-triggered immunity (ETI) (Jones and Dangl, 2006; Harris *et al.*, 2015). However, if either the plant or the pathogen lacks these corresponding genes, the plant will be susceptible to the infection as it will be unable to activate defence responses. ETI is especially effective against biotrophic pathogens (Lukasik and Takken, 2009).

Defence responses can occur rapidly through the oxidative burst, localized cell death, accumulation of phytoalexins, synthesis of pathogenesis-related (PR) proteins and cell wall strengthening proteins (hydroproline-rich glycoproteins). They could also enhance transcription of genes encoding enzymes such as peroxidases, lipooxygenases, superoxide dismutase and phenylalanine ammonia lyase (PAL) involved in the flow of carbon from the primary metabolism into the secondary metabolites (Tanaka *et al.*, 1989). PAL is a key enzyme in the biosynthesis of phenolic compounds that have antimicrobial activities (Flors *et al.*, 2005; Torres *et al.*, 2006). Delayed defence responses following further colonization by the pathogen occur through the plant recognising conserved microbial features such as flagellin, chitin, glycoproteins or lipopolysaccharides (exogenous) generally referred to as pathogen-associated molecular patterns (PAMPs) (Jones and Dangl, 2006; Heil, 2009).

Endogenous plant elicitors are also released following tissue damage and are referred to as damaged-associated molecular patterns (DAMPs) which mediate defence responses to both pathogens and herbivores (Jones and Dangl, 2006; Heil, 2009). Both PAMPs and DAMPs are recognized by plasma membrane-localised recognition receptors (PRRs) (Huffaker *et al.*, 2006; Miya *et al.*, 2007). An immune response triggered by these defence elicitors is known as PAMP-triggered immunity (PTI, previously called basal resistance) with its key component being HR in the form of localised cell death at the site of pathogen entry (Mur *et al.*, 2008).

ETI is often associated with the accumulation of reactive oxygen species (ROS) and the activation of diverse groups of defence-related genes including several families of pathogenesis-related (PR) proteins (Ferreira *et al.*, 2007). A few hours to several days after HR development, the un-inoculated portions of the plant often display increase levels of PR gene expression. This leads to the development of systemic acquired resistance (SAR) which is a broad-based and long-lasting resistance to a wide range of pathogens (Boller and Keen, 1999).

1.3.3.1 Host-Pathogen Recognition

Plants possess pre-formed physical barriers that make them naturally resistant to most pathogens. However, pathogens can bridge these natural defences thus making the plant susceptible (Dangl and Jones, 2001). The plant-borne disease resistance (R) gene and the pathogenic avirulence (*avr*) gene are involved in the interaction between resistance or susceptible plants and the pathogen (Figure 1.2). The interaction of these genes was first described by Flor (1971) to exist in a gene-for-gene manner. Flor hypothesised that for every incompatible reaction, the resistant plant contains an R gene while its corresponding *avr* gene resides in the invading pathogen. Therefore, if either R or *avr* gene is absent in either host or pathogen, disease ensues while if both are present, the host plant becomes resistant (Flor, 1956). This interaction will be observed by a rapid localised cell death at the site of infection which forms part of the HR (Mur *et al*., 2008). Following the HR, defence can be activated in distal uninfected cells giving rise to the systemic acquired resistance (SAR) which enables the plant to be resistant to a large array of other pathogens for longer periods (Boller and Keen, 1999). The HR could also result in the accumulation of PR (Dempsey *et al*., 1999).

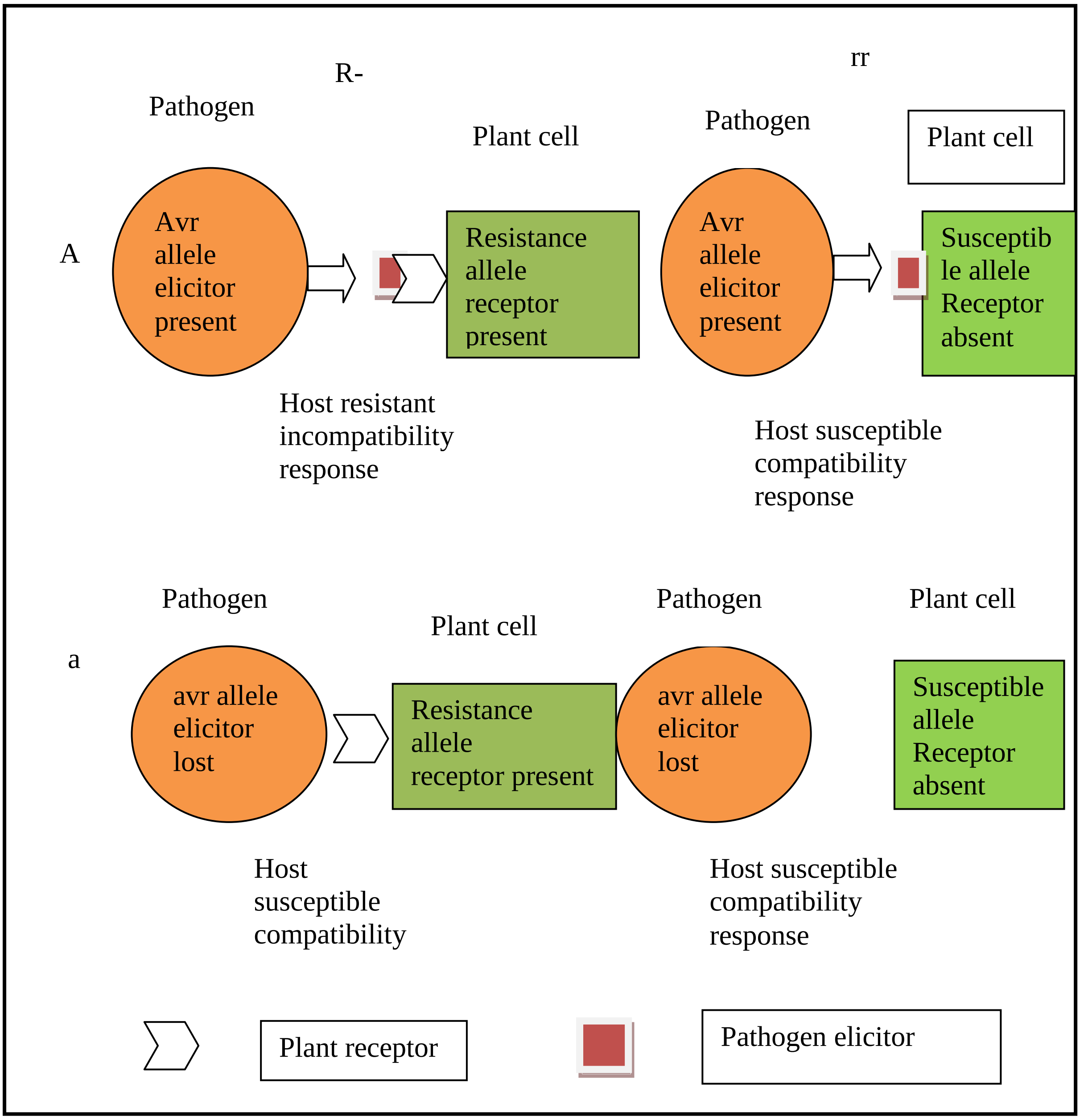


Figure 1.2: Flor’s gene-for-gene model (Adapted from Hammond-Kosack and Jones, 2000).

1.3.3.2 The Hypersensitivity Response

The hypersensitive response (HR) results from the incompatibility between a host plant and a specific pathogen characterised by localised cell death in close proximity to the infection site (Morel and Dangl, 1997). At the necrotic lesions, antimicrobial compounds could be released with induction of intense metabolic alterations in the surrounding cells (Morel and Dangl, 1997; Heath, 2000). Necrosis effectively restricts the fungal pathogen from spreading and also impedes nutrient uptake by biotrophic fungi. Pathogen identification occurs when the avirulence gene products secreted by the pathogen bind to or indirectly interact with the product of a plant resistant (R) gene in a gene-for-gene model (Flor, 1956).

In the first phase of the HR, activation of the R genes triggers an ion influx which involves the efflux of hydroxide and potassium out of the cells and an influx of calcium and hydrogen ions into the cell (Ben, 2007). In the second phase, the cells involved generate an oxidative burst through the production of reactive oxygen species (ROS, superoxide anions, hydrogen peroxide, hydroxyl radicals and nitrous oxide) all affecting cellular membrane function by inducing lipid peroxidation and damage (Laloi *et al.*, 2004; Ben, 2007). During HR, infected plants induce cell death in those cells surrounding the site of infection thus encircling the invading pathogen with a layer or a ring of dead cells thereby inhibiting its growth (Coll *et al.*, 2011). An infected plant also strengthens its cell walls by the deposition of different phenolic compounds while synthesising diverse toxic compounds (phytoalexins and PRs) (Coll *et al.*, 2011). HR also involves the generation of salicylic acid (SA), jasmonic acid (JA), ethylene, nitric oxide, oxylipins, benzoic acids which induce overlapping signalling pathways resulting in the activation of molecules and the expression of defence enzymes/small molecules (Harris *et al.*, 2003).

1.3.3.3 Reactive Oxygen Species (ROS)

Plants naturally produce ROS (by-products of successive reductions of molecular oxygen) as signalling molecules to control various processes including pathogen defence, programmed cell death (PCD) and stomatal behaviour (Apel and Hirt, 2004). The major forms of ROS are superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (HO^-) which are highly reactive and toxic, therefore plants maintain them at lowest levels by a protective mechanism involving the production of superoxide dismutase, peroxidase and catalase (Bolwell and Wojtaszek, 1997). Under stress conditions, this protective action is overcome by the rapid production of large amounts of ROS reaching intracellular concentrations of up to 1M hydrogen peroxide in about 13 minute in a process known as the Oxidative burst (Jacks and Davidonis, 1996). Avirulent pathogens are recognised by the R gene products in the plant immune system which elicits a biphasic ROS accumulation with a low-amplitude, transient first phase, followed by a sustained phase of much higher magnitude that correlates with disease resistance (Lamb and Dixon, 1997). The rapid generation of superoxide and accumulation of H_2O_2 following perception of pathogen avirulence signals is a characteristic feature of the HR, resulting in cell death thus preventing the spread of the pathogens (Ben, 2007).

Several mechanisms have been associated with the production of ROS in response to infection although the involvement of NADPH oxidase which seems to be predominant in most cases (Torres *et al*., 2005; Toress, 2010). In this NADPH-dependent oxidase system the plasma membrane localised NADH oxidases (NOX) catalyses the production of O_2^- by the reduction of oxygen (Torres and Dangl, 2005). Amongst the ROS, H_2O_2 is the most stable and easily penetrates membranes thereby serving as signalling molecules for defence responses in surrounding cells as well as a substrate for oxidative cross-linking in the plant cell wall leading to the direct killing of pathogens (Veal *et al*., 2007). H_2O_2 seems to be the diffusible signal for the induction of cellular protectant genes in the surrounding cells including the PR proteins and the establishment of SAR (Dempsey *et al*., 1999). Activation of the oxidative burst is a central component of a highly amplified and integrated signalling system involving SA production and perturbation of cytosolic Ca^{2+}, which underlines the expression of disease-resistant mechanisms (Lamb and Dixon, 1997).

1.3.3.4 Systemic acquired resistance (SAR)

Avirulent pathogens do not only trigger defences locally, they also induce the production of signalling molecules such SA, ET, and JA. This leads to the systemic expression of antimicrobial PR proteins in the uninfected distal tissue that protects the rest of the plant from secondary infection (Durrant and Dong, 2004). This is known as SAR which is a broad-spectrum response with no specificity to the initial infection and protects the plant from secondary infection for long periods (Fu and Dong, 2013). An important component of SAR is the PR genes which encode PR-proteins that accumulate in the apoplast (extracellular) and the vacuole (intracellular). These proteins include β-1, 3-glucanase, chitinase and peroxidise;

with some showing *in-vitro* antimicrobial activity (Morrisey and Osbourn, 1999). Chitinases and *β* -1, 3-glucanases are the two most important hydrolytic enzymes which are predominant in many plant species after infection by different pathogens. They degrade fungi cell walls as β -1, 3-glucan and chitin are the major structural components of cell walls of many pathogenic fungi. Beta -1, 3-glucanases are co-ordinately expressed along with chitinases after fungal infection with their production associated with many plant species including wheat. PR proteins accumulate at primary infection sites as well as in tissues showing SAR and prevent infection of plant cells (Stintzi *et al*., 1993).

1.3.3.5 Induced systemic resistance (ISR)

Different treatments of plants can boost the basal defence response thus allowing a stronger and faster activation of the defence during subsequent pathogen attack (Frost *et al.,* 2008, Cawood *et al*., 2013). This defence boosting is known as priming and includes SAR that is associated with SA and ISR (Figure 1.3) (Loake and Grant, 2007; Cawood *et al*., 2013). ISR could be mediated by biotic agents such as plant growth promoting rhizobacteria (Van Wees *et al*., 2008). ISR differs from SAR in that it mainly functions independently of SA although it involves the JA and ET signalling pathways (Kessler *et al*., 2006; Ton *et al*., 2009). Several synthetic and natural plant activators have been used to induce resistance in wheat to infection. When acibenzolar-S-mehyl (a synthetic plant activator BTH) was applied to wheat, it led to an increased resistance against powdery mildew, leaf rust and leaf spot (Gorlach *et al*., 1996).

A previous study conducted by Cawood *et al.* (2010) revealed that foliar application of a crude extract *Agapanthus africanus* resulted in an increased PR protein activity and a reduction in disease severity during subsequent infection with *P. triticina*. Cawood *et al.* (2013) in another study, reported that pre-treatment of both infected resistance and susceptible wheat with the crude extract of *Agapanthus africanus* resulted in an increase in β-1, 3-glucanase levels. A greater expression of PR3 and PR9 genes was also observed in infected and susceptible seedlings. This led to the conclusion that the induced expression of all the defence-related genes was as a result of the crude *A. africanus* extract priming the resistance response of wheat prior to the infection.

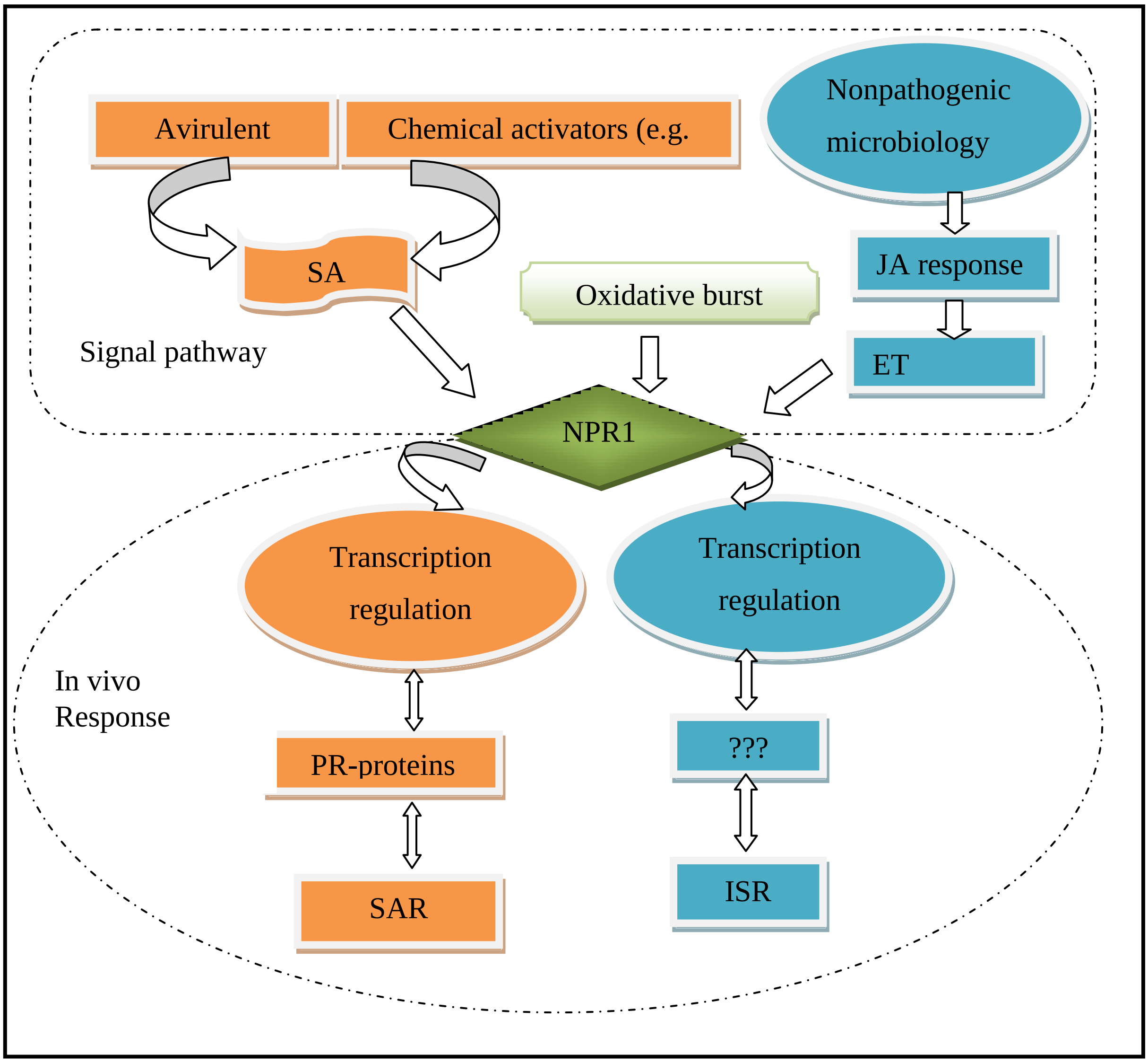


Figure 1.3: Signal transduction pathways for SAR and ISR (Adapted from chan, 2013).

1.1.4 Resistance mechanism of wheat to rusts

Approximately 10% of crop yield is reduced by rust diseases in more developed agricultural systems as opposed to a greater than 20% in the less developed (Dangl *et al.*, 2013). To counter possible losses, disease resistance breeding has been a key component of crop improvement programmes from the time when Biffen first established single gene inheritance of resistance to *Puccinia striiformis* in wheat (Biffen 1905). However, from this initial breeding programme focused on major gene resistance to protect crops from specific races of pathogens, race-non-specific resistance has been discovered and is now being included in breeding strategies (Ellis *et al.*, 2014). Cultivars of wheat, resistant to rusts, have been developed over the years; however the precise mechanism of the resistance remains largely unknown. Resistant and susceptible wheat cultivars react differently at the genomic level and consequently at the cellular level through changed cellular functions. In contrast to susceptible plants, resistant plants either partially or completely suppress pathogen growth or replication at the site of infection. Although this is not always the case, one of the most visible phenotypes that is frequently associated with plant resistance is the rapid localised cell death that results from the hypersensitivity reaction (HR) at the site of infection (Mur *et al.*, 2008). It is observed on disease resistant plants by the appearance of chlorotic and/or necrotic flecks resulting from the interaction between resistant genes in the host and the corresponding avirulence genes in the pathogen (Mur *et al*., 2008). Other resistance responses may include but are not limited to activation of defence gene expression, leading to production of antimicrobial proteins or low molecular weight antibiotics (Heath, 2000). A pathogen's ability to infect and cause disease in its host plant is referred to as virulence while its inability to infect a resistant genotype of its normal host is called avirulence (Ellis *et al*., 2014).

Plant resistance to a pathogen can be orchestrated by a single gene differentiating a resistant host genotype from a susceptible with the genes referred to as resistance genes. It has been observed that cuticle and cell wall thickness influence resistance to certain pathogens by reducing the ability of the pathogen to enter via the thick and tough cell walls (Freeman and Beattie, 2008). A common response of plants to fungal attack is the deposition of a callose, (1, 3)-β glucan polymer in the form of cell wall thickening called papillae at the site of penetration. According to Ellinger *et al*. (2013), the deposition of callose in elevated amounts at an early stage of infection could strongly support penetration resistance. Although the function of most compounds has been directly linked to cell wall re-enforcement or anti-microbial effect, the role of callose remains unclear (Voigt, 2014). Callose deposition may favour the pathogen by contributing to the stability and function of the haustoria. It acts as a barrier that makes haustoria less susceptible to toxic metabolites that are produced by the host and accumulate in the infection site (Jacobs *et al*., 2003). On the other hand, callose may limit the diffusion of pathogen-derived elicitors, thus reducing the activation of defence responses (Underwood, 2012).

1.5 RUSSIAN WHEAT APHID (RWA)

1.5.1 Introduction

Aphids are known to be the largest group of phloem-feeding insects and their enormous reproductive potential that allows quick infestation of their host makes them one of the most devastating pests to crop production especially wheat and barley (Davis, 2012; Botha *et al*., 2014). The Russian Wheat aphid (RWA), *Diuraphis noxia* (Mordvilko) belongs to the family *Aphididae* which comprise of more than 4300 species specialised to feed on phloem sap (Douglas, 2006). *D. noxia* populations in South Africa, as well as many international locations, reproduce through facultative parthenogenesis, unlike in areas where it is endemic and can also reproduce sexually (De Jage *et al*., 2014).

1.5.2 Wheat resistance and Russian wheat aphid Biotypes

The destructive nature of *D. noxia* on crops has resulted in the putting in place of several strategies to control the pest. The primary control mechanism being the use of chemicals such as pesticides, biological agents by means of introducing natural enemies and agricultural measures which include the use of agronomic practices such as planting dates, early maturing cultivars and crop rotation (Peairs *et al*., 2006; Tolmay *et al*., 2007; Hajek *et al*., 2007; Botha *et al*., 2014). The efficacy of biological control can be enhanced by coupling it with resistant genotypes; since resistant cultivars exhibit less leaf rolling which therefore provides predators and parasitoids with better access to developing aphid colonies (Jyoti and Michuad, 2005; Khan *et al*., 2013). Genetic plant resistance to RWA is also considered a more desirable alternative to the use of expensive, toxic and environmentally hazardous chemicals (Tolmay *et al*., 2007). Plant resistance is therefore a defensive strategy against insects which results in a reduction of crop damage. Numerous sources of resistance to *D. noxia* have been identified in members of the Triticeae family and are used extensively in the breeding of resistant cultivars (Dogimont *et al*., 2010). There are resistant and non-resistant (susceptible) wheat varieties available to South African farmers.

Biotypes are insect populations (containing resistant genes) which are able to kill or injure a plant. The presence of these resistant genes complicates the pest management programs that are based on host plant resistance (Shufran and Payton, 2009). Environmental factors that reduce reproductive success in the aphids contributes to evolutionary changes (selective pressure) resulting in the emergence of new biotypes (Sugio *et al*., 2014; Bruce, 2015). The emergence of new biotypes renders current resistant cultivars susceptible in areas where it occurs (Jankielsohn, 2014). The differences in the aphid biotypes depend on the resistant genes present in the wheat cultivar and the biotype that can overcome it. Therefore wheat cultivars with resistant gene *Dn*1 could be resistant to RWA SA1 but susceptible to RWA SA2, RWA SA3 and RWA SA4 (Jankielsohn 2014). Furthermore, a high degree of biotype diversity has since been reported in worldwide collections of RWA (Puterka *et al*., 1993). The RWA biotype classification was first initiated by Puterka and colleagues who used a series of wheat, barley and rye differentials to characterise a world collection of aphids with seven biotypes reported worldwide in the early nineties (Puterka *et al*., 2014)

In South Africa, currently four Russian wheat aphid biotypes (Table 1.1) have been collected and identified (Jankielsohn, 2014). The first reported was in 1978 and the biotype was designated as RWA SA1 (Du Toit and Walters, 1984). Later on in 2005, biotype RWA SA2, virulent against the *Dn*1 resistant gene was recorded in wheat producing areas, especially in the Eastern Free State (Tolmay, *et al*., 2007). RWA SA3, virulent against the *Dn*4 resistant gene, was recorded in 2009, also predominantly in the Eastern Free State (Jankielsohn, 2011). Recently, RWA virulent against the *Dn*5 resistant gene designated as RWA SA4 has been reported near Bethlehem in the Eastern Free State (Jankielsohn, 2014).

Table 1.1: Summary of South African RWA biotypes

Biotype	**Year identified**	**Virulent against**	**Reference**
RWA SA1	1978	*Dn*1	Du Toit & Walters, 1984
RWA SA2	2005	*Dn*1, *Dn*2, *Dn*3 & *Dn*9	Jankielsohn, 2011
RWA SA3	2009	*Dn*1, *Dn*2, *Dn*3, *Dn*4 & *Dn*9	Jankielsohn, 2011
RWA SA4	2011	*Dn*1, *Dn*2, *Dn*3 *Dn*4, *Dn*5 & *Dn*9	Jankielsohn, 2014

1.5.3 RWA resistant genes and resistance mechanism

Plants are attacked by a variety of insects and pathogens and have therefore needed to develop defence strategies which are regulated by phytohormones (Jankielsohn, 2013; Lazebink *et al.*, 2014). This has resulted in the selection of insect biotypes which have evolved particular resistance giving rise the perceived arms race between the insects, pathogens and plants (Sugio *et al.*, 2014). Since the new biotypes exert a selection pressure on the host plant, those plants which have developed a new defence strategy will survive thereby resulting in the development of new resistance in the plant (Sugio *et al.*, 2014; Bruce, 2015). Consequently, the survival of the insect is reduced and this again results in the development of counter-resistance in the insect. By breeding resistant lines, breeders speed up the natural process in favour of the plant, however, when resistant cultivars are released, counter-resistance of the insect or pathogen still occurs, thereby necessitating the constant development of new resistant cultivars. RWA resistant cultivars have been bred throughout the world and therefore numerous commercially resistant cultivars are available to farmers (Tolmay *et al*., 2007).

Host plant resistance is the base of cereal insect pest management programs with several *D. noxia* resistance (*Dn*) genes introduced in commercial cultivars to manage aphid infestation (Khan *et al*., 2013; Puterka *et al*., 2015). The advantage of resistant cultivars to producers is a direct economic benefit observed by a reduction in the cost of pesticides and fuel owing to fewer applications. The deployment of genetic resources for resistance also assists in achieving yield stability without resorting to harmful chemicals. In addition, it prevents environmental degradation and benefits the producers who cannot afford the use of costly chemicals (Marasas, 1999). In South Africa, host plant resistance in wheat has been introduced and applied successfully as a control measure against RWA (Van Nierkerk, 2001; Tolmay and Van Deventer, 2005). These resistant cultivars form the basis of an integrated-control programme which was developed in the country by the Small Grain Institute (SGI) of the South African Agriculture Research Council (ARC) (Hatting *et al*., 2004).

Resistance orchestrated by various *Dn* genes, could be phenotypically categorised as antibiosis, antixenosis or tolerance, although some cultivars may exhibit a combination of these (Smith *et al*., 1992; Haile *et al*., 1999). For example cultivars with the *Dn*5 gene exhibit a combination of antibiosis and antixenosis (Marauis and de Toit, 1993; Wang *et al*., 2004;

Heyns *et al*., 2006). In antibiosis, the plant restricts the aphid's rate of increase by affecting its biology thereby reducing the reproductive fitness of the insect and it is the *Dn*1 gene that is responsible (Wang *et al*., 2004; Botha *et al*., 2014). In antixenosis (non-preference) the resistant cultivar defers aphids from settling and colonising the plant. The *Dn*2 gene has been characterised to impart tolerance to RWA whereby the wheat plants survive but are stunted in growth (Wang *et al*., 2004). Resistance in some wheat lines appear to be mostly from antibiosis with some tolerance (Du Toit, 1989; Quisenberry and Schotzko, 1994). A resistant wheat line could show moderate tolerance, high antibiosis, and little antixenosis (Baker *et al*., 1992) while other lines are tolerant with low levels of antibiosis (Smith *et al*., 1992). Studies have shown that available resistant wheat cultivars are highly variable in their capacity to retain yield potential under severe *D. noxia* infestations (Tolmay and Deventer, 2005). There is therefore a potential for the development of resistance-breaking aphid biotypes which necessitates the continuous development and integration of biocontrol agents in pest management programmes.

1.5.4 Plant response to attack by insect herbivores

Over the years, plants have evolved sophisticated systems to cope with herbivore challenges (Saheed *et al*., 2008; Wu and Beldwin, 2010; Boyd, 2012). Plants are able to perceive herbivore-derived physical and chemical signals such as elicitors in the insect's saliva as well as compounds in oviposition fluids and dramatically reshape their transcriptomes, proteomes and metabolomes. All these herbivory-induced changes are mediated by elaborate signalling networks including receptors, Ca^{2+} influxes, kinase cascades, ROS and phytohormone signalling pathways (Laxalt *et al*., 2007; Wu and Baldwin, 2010; Mur *et al*., 2013; Kanchiswamy and Maffei, 2015). Furthermore, herbivory induces defence responses not only in the wounded regions but also in the undamaged regions of the attacked leaves as well as in distal intact (systemic) leaves (Wu and Baldwin, 2010). Plants can employ two general strategies to defend themselves against herbivory; they can either reduce the amount of damage they experience (resistance) or tolerate the damage (Kessler and Baldwin, 2002). Therefore resistance is a heritable trait contained by a plant species and reduces insect populations in the field. Upon gene activation, plants can defend themselves directly or indirectly (Mithofer and Boland, 2012).

Direct plant defences involve a vast array of chemicals including proteinase inhibitors which enhance plant resistance to insects by actively inhibiting their proteolytic digestive enzymes; polyphenol oxidase and also toxic compounds such as alkaloids, terpenoids and phenolics that are poisonous to broad-spectrum herbivores (Kessler and Baldwin, 2002). Marcio and Silva-Filho (2002) classified plant toxic compounds into chemical-derived substances and protein-derived molecules (Table 1.2). Plant defensive chemicals are either constitutive or induced. Constitutive substances prevent insect feeding by direct toxicity or reduced the digestibility of plant tissue by the insect whilre inducible substances are synthesised in response to tissue damage (Marcio and Silva-Filho, 2002) (Figure 1.4).

Indirect plant defence mechanisms against herbivores rely on the release of volatile organic compounds (VOCs) (Shiojiri *et al*., 2012). The VOCs either attract predators or parasitoids of the herbivores or result in the upregulation of thykaloids in adjacent plants making them unpalatable to the herbivore (Birkett *et al*., 2000; Dangl and Jones, 2001; Shiojiri *et al*., 2012). VOCs are mainly C6-alcohols and –aldehydes, terpenes, ethylene and molecules derived from shikimate. Trichomes density could also act a defence mechanism against herbivory forming a physical barrier to *D. noxia* feeding. Bahlman *et al*. (2003) observed that trichomes in resistant Tugela-*Dn*1 plants were 1.7 times greater than in the susceptible Tugela line.

Plant damage by chewing insects usually elicit a wound response mediated mainly by ethylene (ET) and Jasmonic acid (JA) (Howe and Jander, 2008; Acosta and Farmer, 2010). Plants response to aphids feeding via piercing-sucking mouthparts differs from that elicited by the more mechanical damage inflicted by other insects with chewing mouthparts, as they exhibit a more intimate and sophisticated mode of biotrophic parasitism (Van de Westhuizen *et al*., 2002). The biochemical origin of wheat resistance to *D. noxia* is still relatively unclear. However, since the aphid probes mainly intracellularly before the stylet penetrates the phloem; several defence-related products have been shown to accumulate in the apoplast of resistant wheat including pathogenesis-related proteins (chitinases) and oxidative enzymes (peroxidases) (Van de Westhuizen *et al*., 1998; Morkumas *et al*., 2011). However resistance is not constitutively expressed and is induced by aphid infestation, therefore the level of response varies in different resistant cultivars and the genetic background in which *Dn* gene is bred plays an important role in the effectiveness of the resistance response (Botha *et al*., 2005).

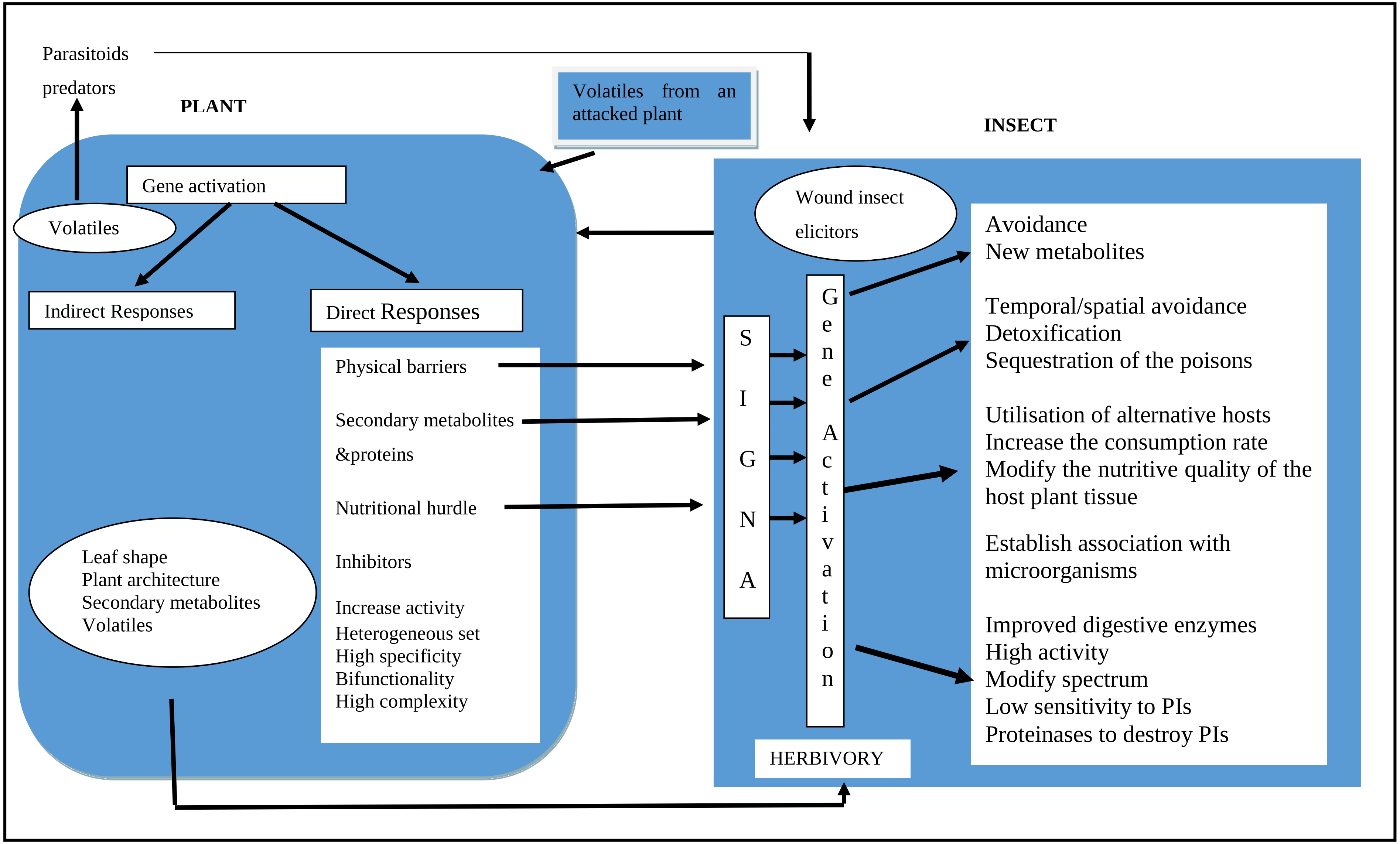


Figure 1.4: Plant interactions with insect (Adapted from Marcia and Silva –Filho, 2002).

Table 1.2: Chemical derived substances and protein derived molecules involved in host-plant resistance to insects (Adapted from Marcia and Silva-Filho, 2002).

Class/Subclass	**Function**
A: Chemical-derived substances	
Alkanes, aldehydes, ketones, waxes	Protective layer
Lignins, tannins	Mechanical barrier, unpalatability, post absorptive inhibition
Terpenoids (monoterpenoids, iridoids, sesquiterpenoids, diterpenoids, triterpenoids)	Toxicants, antibiotic, feeding dterrents, oviposition deterrents
Phenolics	Toxicants, ovicidal, photosensitizing insects
Flavonoids (anthocyanins, flavonols, flavones, isoflavonoids)	Antifeedant, phytoalexins, inhibition of mitochondrial oxidation
Quinones	Toxicant, antifeedant
Alkaloids	Toxicants, interfere in the nerve system, digestive enzyme inhibitor, antifeedant, glucosidase inhibitor
Cyanogenic glycosides	Toxicant
Glucosinolates	Repellent, toxicant, irritant, antibiotic
B. Protein derived molecules	
Lectins	Interfere in the absorption of nutrients, increase the absorption of toxic substances
Chitinases	Damage the insect midgut
α-amylase inhibitors	Digestive enzyme inhibitor
Proteinase inhibitors	Digestive enzyme inhibitors
Indole-3-glycerol phosphate lyase (IGL)	Formation of free indol
Vegetative storage protein (VSP)	Part of the systemic response
Glutathione S-transferase (GST)	Detoxify or inactivate toxic compounds
β-glucosidase 1 (BGL1)	Unknown
Calcium binding elongation factor (CaEF)	Signalling pathway
Hevein-like protein (HEL)	Unknown
Phospholipase A2	Generation of second messenger
MAPkinase	Phosphorylation of transcription factors
Polyphenol oxidade (PPO)	Reduction of the nutritive value of protein
1-aminocyclopropane-1-carboxylic acid oxidase (ACO)	Ethylene biosynthesis
Allene oxide synthase (AOS)	JA biosynthesis
Phenylalanine ammonia-lyase (PAL)	Phenylpropanoid pathway
Peroxidase	Lignin synthesis, hypersensitive response
Lipoxygenase (LOX)	JA biosynthesis

1.6 Plant stress response pathways

Plants in their natural habitat encounters multiple biotic and abiotic challenges simultaneously that could reduce their productivity (Rojeb *et al*., 2014). Plants are able to identify and prioritise each signalling pathway to mount the most efficacious defence strategy to minimise damages caused by insects or pathogens (Xu and Brosche, 2014). Defence responses against insects and pathogens are regulated differentially by cross-communicating signalling pathways in which Salycilic acid (SA), Jasmonic acid (JA) and Ethylene (ET) play key roles (Bostock, 2005; de Vos *et al*., 2005; Spoel and Dong, 2008). These secondary signalling molecules can activate both local and systemic defences. The plant synthesises new enzymes and metabolites that are chanelled into newly activated biosynthesis pathways (Singh *et al*., 2007). Chitinases and β-1, 3-glucanases are amongst the enzymes that respond to pathogen and degrade pathogen cell walls as well as serving as antimicrobials. Signalling interactions could either be mutually antagonistic or synergistic resulting in negative or positive functional outcomes. Cross talk helps the plant to minimize energy costs and creates a flexible signalling network that allows the plant to fine tune its defence response to the invaders encountered (Pieterse *et al*., 2001; Bostock, 2005).

In Arabidopsis plants, de Vos *et al*. (2005) revealed that the signal signature in each plant-attacker combination and the kinetics of SA, JA and ET production vary greatly in both quantity and timing. Plant responses to herbivore attack are complex and genes activated upon attack are strongly correlated with the mode of herbivore feeding and the degree of tissue damage at the feeding site (War *et al*., 2012). Aphids produce little injury to plant foliage and are perceived as pathogens thereby activating the salicylic acid (SA)-dependent and Jasmonic acid (JA)/ethylene-dependent signalling pathways (Arimura *et al*., 2009). On the other hand, four signalling pathways are important in response to pathogens including SA; the ROS; and the JA/ethylene-dependent pathways. Necrotrophs activate Jasmonic acid (JA) and ethylene dependent responses whereas biotrophic pathogens activate the salicyclic acid (SA) dependent response (Lukasik and Takken, 2009; Rahman *et al*., 2012). Although the plant has placed these mechanisms in place, the insect herbivores and pathogens have also evolved to manipulate plants for their own benefit by suppressing induced defences through modulation of the plant’s defence-signalling pathways (Pieterse and Dicke, 2007).

1.6.1 The salicylic acid pathway

The SA-dependent cascade uses SA, a plant phenolic and its methyl conjugate (MeSA) to stimulate expression of defence-response genes, including PR genes that encode proteins with an apoplastic localisation (acidic PR genes) (Mayer *et al*., 2002). It has also been reported that SA promotes the development of SAR, which confers a broad-range resistance to pathogens and some insects (Dampsey *et al*., 1999; Delaney, 2000). Salicylic acid is crucial for the localised HR (Aviv *et al*., 2002) (Figure 1.5). Although SA induction and related compounds are typically associated with plant defence against pathogens, studies in recent past have shown that sap-sucking insects also trigger the accumulation of SA in wheat (Chaman *et al*., 2003) as well as in barley (Zimmerli *et al*., 2004). Van der Weshiuzen et al. (1998) showed that the induction of downstream defence related enzymes, β-1, 3-glucanse, chitinase and peroxidase in resistant wheat plants was responsible for wheat resistance against RWA.

SA can antagonise the JA-signalling pathway and vice versa. In this pathway the non-inducible pathogenesis-related protein 1(NPR1) is considered the master regulator and interacts with the TGA transcriptome factors, leading to the activation of the SA-dependent responses (Rahman *et al*., 2012). Following infection, levels of SA increase in plant tissue and exogenous application of SA has resulted in enhanced resistance to a wide range of pathogens (Ryals *et al*., 1996). Glazebrook (2001) revealed that SA is involved in the regulation of many PR genes including those encoding *PR*1, *PR*2 and *PR*5 and are required for the rapid activation of defence responses and the establishment of SAR. The role of SA which is now considered as a hormone-like endogenous regulator has long been established. Chen *et al*. (1993) and Conrath *et al.* (1995) demonstrated that SA binds and inhibits tobacco catalase activity both *in-vitro* and *in-vivo*. Inhibition of this catalase, a H_2O_2 scavenging enzyme by SA would result in an increase in endogenous levels of H_2O_2 generated by photorespiration, photosynthesis, oxidative photophosphorylation and the hypersensitive response-associated oxidative burst. The H_2O_2 and other reactive oxygen species derived from it could serve as second messengers to activate the expression of plant defence-related genes such as *PR*-1. SA is predominately involved in defence against phloem-sap-sucking insects and biotrophic pathogens (Zhang *et al*., 2009; Thaler *et al*., 2010; 2012).

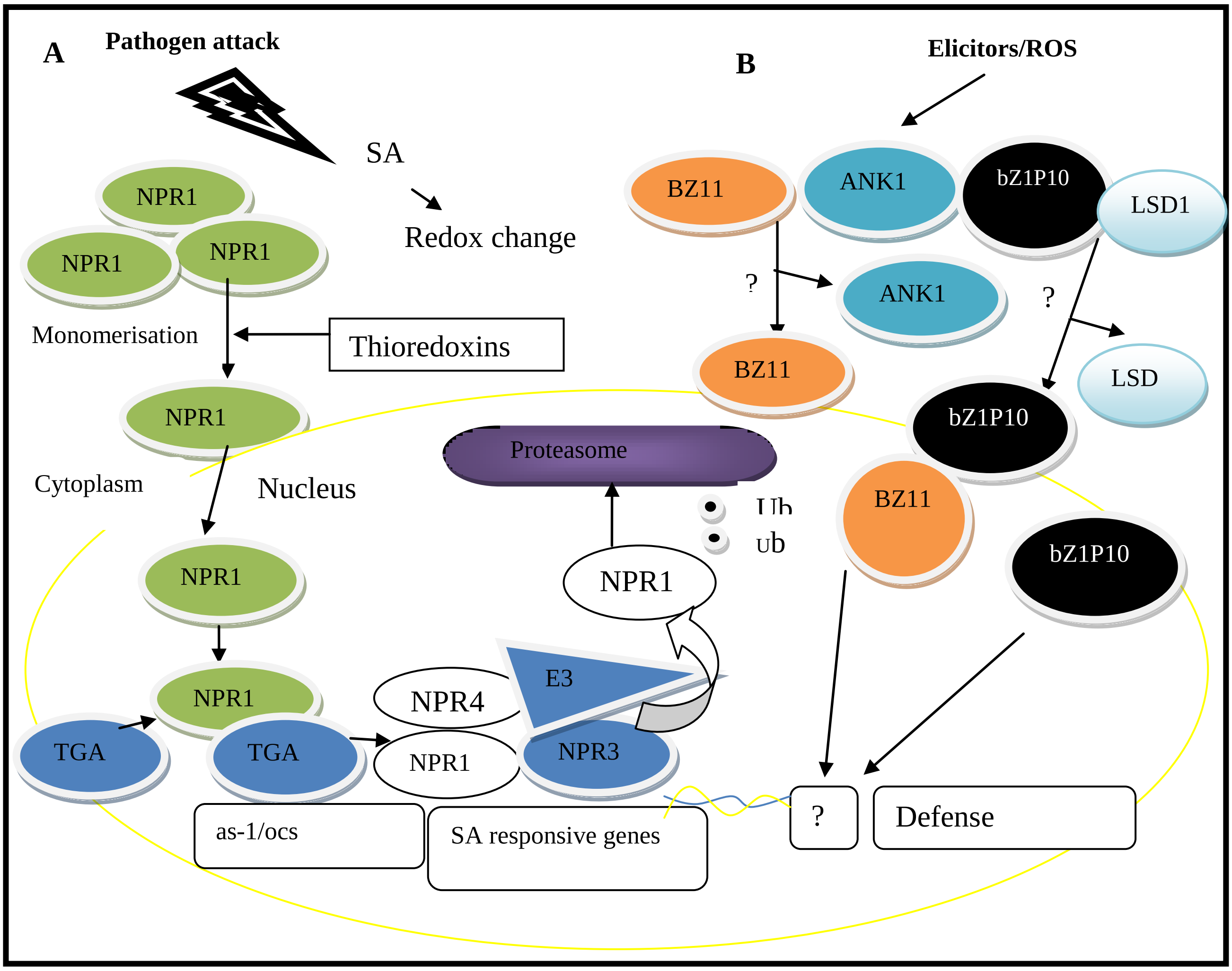


Figure 1.5: Salicylic acid pathway (Adapted from Farmer *et al*., 2003)

1.6.2 Reactive Oxygen Species (ROS) Nitric Oxide (NO) pathway

The second pathway in response to pathogen attack is dependent on the generation of ROS and NO (Figure 1.6). Programmed cell death (PCD) is an integrated cellular process that occurs during plant growth and development and a defence response for the plant to survive various stresses. ROS or NO species mainly O_2^-, H_2O_2 and NO are important in PCD induction. NO, an endogenously produced ubiquitous free radical gas, play an important role in various physiological processes in plants including germination, root development, stomatal closure, hormone signalling and homeostasis (Besson-Bard *et al*., 2008). Nitrate reductase initiates the rapid production of NO in infected cells or when elicited by Microbes-Associated Molecular Patterns (MAMPs) or Damage-Associated Moelcular Patterns (DAMPs) (Rasul *et al*., 2012; Jeandroz *et al*., 2013). Ca^{2+} spiking, Ca^{2+}-binding proteins, protein kinases such as MAPKs, caspase or caspase-like proteases, lipid messengers such as phosphatidic acid and fatty acid hydroperoxides are cellular components for both ROS and NO signalling pathways involved in cell death (Laxalt *et al*., 2007; Mur *et al*., 2013).

These molecules promote HR characterised by tissue destruction at infection site, stimulates SA synthesis and induce some defence-response genes (McDowell and Dangl, 2000). Both molecules could coincidentally occur within the same subcellular organelles such as the mitochondria, in response to stresses with their levels reciprocally controlled or affected by each other through the modification of enzymes involved in synthesis or catabolism (Planchet and Kaiser, 2006). They could also show overlapping and synergistic functions essentially in cell death induction through interactions determined by their reactive nature.

The production balances and diverse interactions between ROS and NO in different environments form a complex signalling network that determines whether a plant cell continues to survive or are directed to death (Zhoa, 2007). 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). Plants control ROS levels and protect cells under stress conditions by expressing ROS scavenging enzymes, such as superoxide dismutase (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).

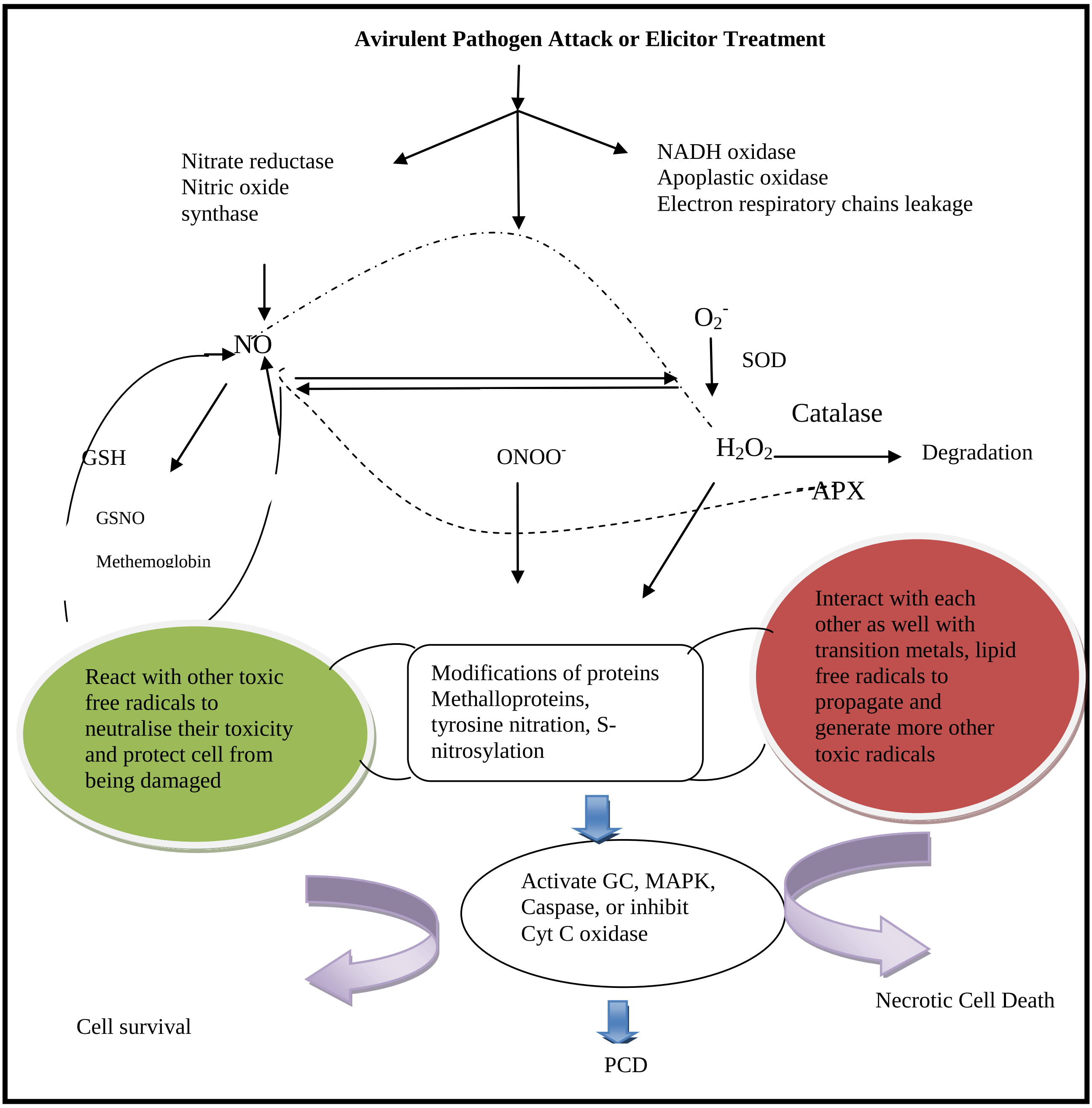


Figure 1.6: Interaction between reactive nitrogen and oxygen species (Adapted from Zhoa, 2007)

1.6.3 The Jasmonic acid/ethylene pathways

Jasmonates, a collective name of Jamonic acid (JA) and its methyl ester, are plant hormones that are key regulators in the development and physiology of plants and are also associated with a wide range of plant defence responses (Pieterse *et al*., 2009; Leon-Reyes *et al*., 2010; Thaler *et al*., 2012). In plant defence responses, jasmonates act as a wound hormone, especially against chewing insects and necrotrophic pathogens, which are able to induce resistance pathways and defence gene expression (Figure 1.7) (Wei *et al*., 2011; Thaler *et al*., 2012). Molecules that have been associated with jasmonate induction include proteinase inhibitors, defence-related volatile compounds and secondary metabolites, such as nicotine, active phenolics and phytoalexins (Balbi and Devot, 2008). The phytohormone ethylene (ET) is a signal molecule for plant development (ripening and senescence), and is involved in the response to biotic and abiotic stress (Wang *et al*., 2002). Production of ET in higher amounts is an early response of plants to the perception of pathogen attack and is related to the induction of defence reactions. It acts in concert with JA as a systemic signal of wound-induced gene activation (Penninckx *et al*., 1998). They both induce a systemic tolerance to a broad range of pathogens called Induced systemic resistance (ISR) which is distinct from systemic acquired resistance (SAR) (Kessler *et al*., 2006; Ton *et al*., 2009). Although ET has no effect on defence-related callose deposition, it has been reported to be involved in several defence responses such as xylem occlusions, cell wall-strengthening by the production of hydroxyproline-rich glycoproteins, phytoalexins and the induction of PR proteins (Adie *et al*., 2007).

Cross talk occurs between the SA and JA/ethylene-dependent signalling pathways. Rises in SA are correlated with down-regulation of the JA/ethylene-regulated defence-response genes as well as JA-regulated wound responses (Glazebrook, 2005; van Wees *et al*., 1999). Studies have shown that simultaneous application of SA and JA at low concentration results in synergistic expression of *PR*1, a classical JA defence marker. In contrast, higher phytohormone concentrations are antagonistic and induce apoplastic reactive oxygen production and cell death (Glazebrook, 2005).

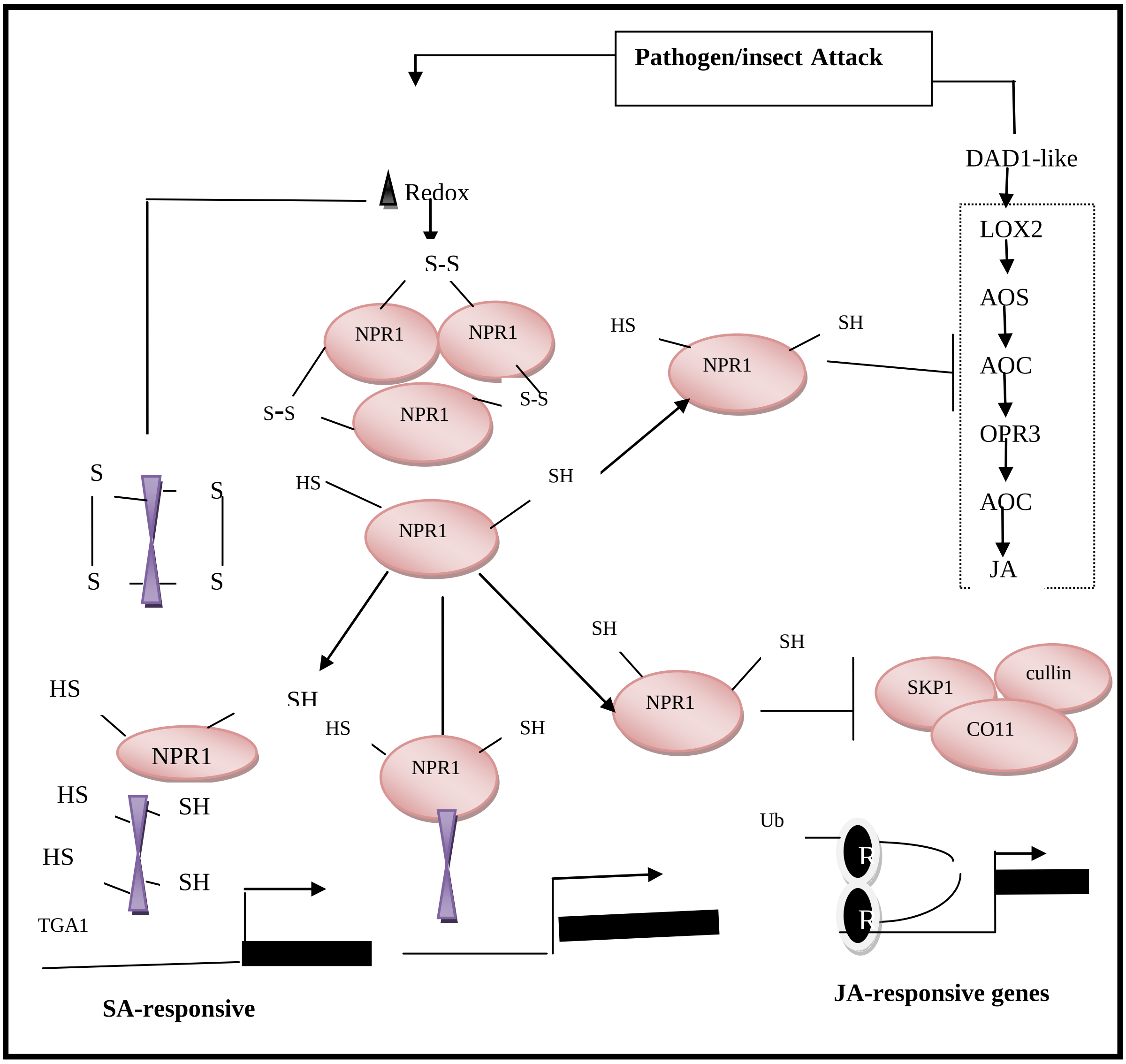


Figure 1.7: The central role of NPR1 in the regulation of SA-induced suppression of JA-dependent defence signaling (Adapted from Koornneef and Pieterse, 2008).

1.6.4 The role of abscisic acid in plant protection

The plant hormone abscisic acid (ABA) has emerged as a key factor in the outcome of plant-pathogen interactions (Figure 1.8). In most cases, ABA behaves as a negative regulator of disease resistance. For instance exogenous application of ABA resulted in increased susceptibility of various plants species to fungal and bacterial pathogens (Achuo *et al*., 2006; Asselbergh *et al*., 2008; Ho *et al*., 2013), on the other hand disruption of ABA biosynthesis conferred resistance to the necrotroph *Botrytis cinerea* (Audenaert *et al*., 2002). In contrast, some studies have shown the positive role of ABA in the activation of defence responses and pathogen resistance. ABA primes callose accumulation enhanced basal resistance in response to *Blumeria graminis* f. sp. *Hordei* and also activates induced resistance in response to the necrotrophic fungi *Altenaria brassicicola* and *Plectosphaerella cucumerina* (Mani-Mauch and Mauch, 2005; Flors *et al*., 2008). ABA has also been shown to play a key role during plant-insect interactions (Flors *et al*. 2009). It is involved in plant defence against insects after aphid infestation of celery plants (susceptible) or sorghum and wheat (resistance) during which several genes that share sequences with those involved in the biosynthesis or that are activated by ABA were upregulated (Divol *et al*., 2005; Boyko *et al*., 2006; Park *et al*. 2006). However, as with plant reactions toward pathogens, ABA has various regulatory functions against insects. Amongst others, several highly upregulated genes under ABA control are involved in cell wall strengthening (Smith and Boyko, 2007).

Callose is a β-1, 3-glucan polymer with β-1, 6-branches, ubiquitous in plants in different tissues and plays diverse roles in plant growth and development. Callose deposition in plasmodesmata is associated with the control of channel conductivity (Simpson *et al*., 2009), whereas when found in sieve tubes in dorminant phloem, they play an important role in sealing them in response to various stimuli (Zavaliev *et al*., 2011). Apart from these physiological activities, callose has been associated with biotic stress responses. Being a major constituent of papillae, it is rapidly synthesized and deposited beneath the attempted site of pathogen penetration as such it is considered an important factor for plant penetration resistance (Huckelhoven, 2007). Pathogenesis-related protein 2 (PR2) plays a principal role in plant defence and in general stress responses. Callose deposition is antagonistic on the SA defence signalling pathway and this suggests that PR2 may function as a modulator of callose- and SA- dependent defence responses (Oide *et al*., 2013).

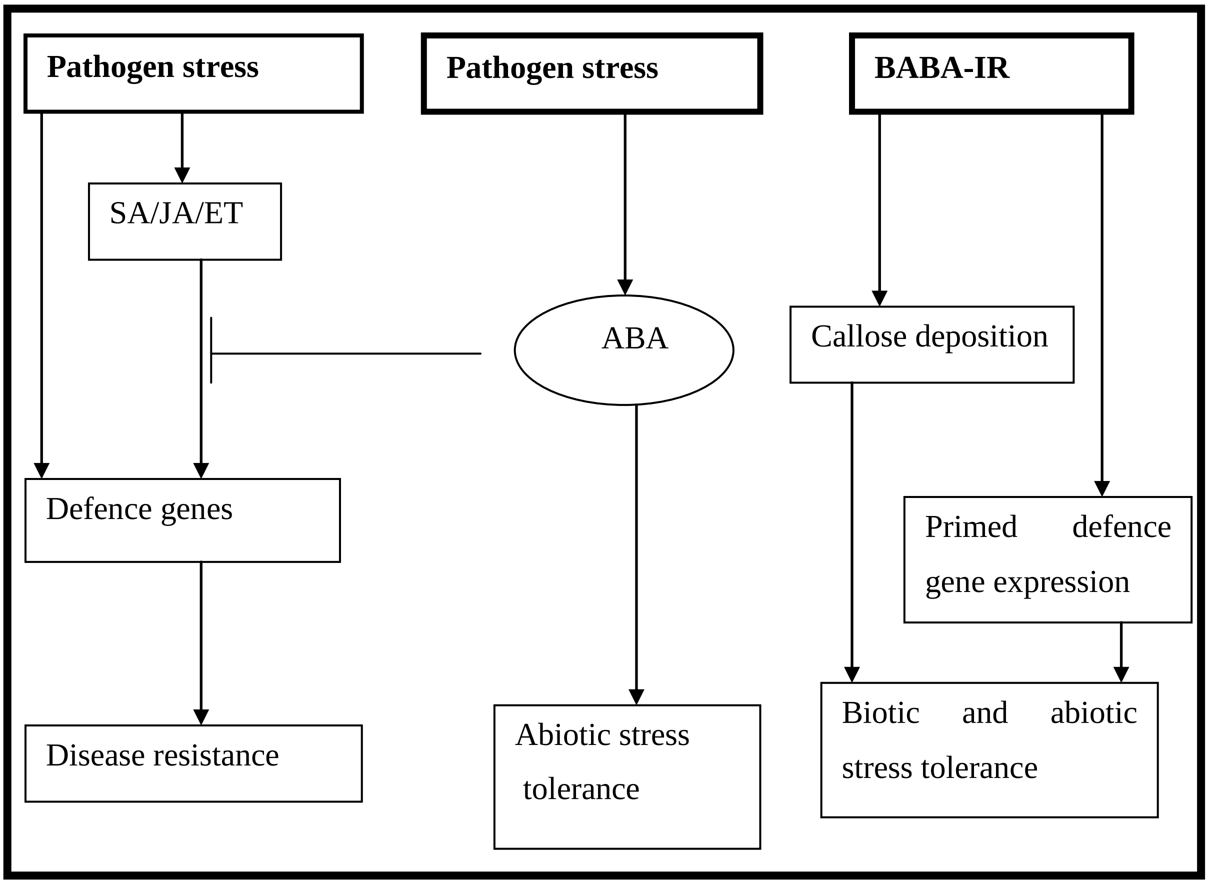


Figure 1.8: The role of abscisic acid in plant defence responses (taking from Mauch-Mani and Mauch, 2005).

1.6.5 Role of β-1, 3 Glucanase

β-1, 3 Glucanases are plants defensive proteins that are abundant in plants and have been characterised from a wide range of species (Balasubramanian *et al*., 2012). This enzyme catalyses endo-type hydrolytic cleavage of the 1, 3-β-glucosidic linkages in -1, 3-glucans. They play key roles in plants including cell division, trafficking of materials through plasmodesmata and defend the planst against fungal pathogens either alone or in conjunction wth chitinases as well as other antifungal proteins (Leubner-Metzger and Meins, 1999; Balasubramanian *et al*., 2012). β-1, 3 Glucanases are grouped in the PR-2 family of pathogenesis-related (PR) proteins. Combining β-1, 3 Glucanases genes with other antifungal genes could be a possible strategy to develop cultivars with durable resistance against fungal (Balasubramanian *et al*., 2012).

1.7 Rationale of the study

Rusts infection and Russian wheat aphid feeding on cereal plants lead to extensive damage and loss of yield (Pretorius and Bender, 2010; Botha *et al.*, 2014). South African wheat farmers spend millions of Rands on pesticides, herbicides as well as on fungicides each year to produce two million tons of cereals. The discovery of new rust races and RWA biotypes pose a serious threat to the world wheat industry including South Africa since the rust and RWA resistant cultivars, which offered a long-term solution in the control of the pathogens and pests, may no longer be effective. The new races and fast evolving biotypes largely shorten effective periods of the existing resistance of cultivars in the agro economic systems. The population of South Africa is expected to reach 55 million by 2020 (http://populationpyramid.net/South). To meet the basic nutritional requirements of the ever-growing population, the agricultural sector has stepped up production of stable food. Wheat is one of the most important stable foods and the third highest produced field crop produced in South Africa, with local production expected to increase. Moreover, the wheat industry is challenged by a number of pests and pathogens (Terefe *et al*., 2007; DAFF, 2013). In order to prevent crop losses due to these pathogens and pests, the country has embarked on breeding cultivars that are resistant to rusts and aphids (Du Toit, 1988; Pretorius *et al*., 2007). However, the breeding process is time consuming and may take up to 25 years before a new cultivar becomes commercially available as breeders select adapted crop varieties traditionally, based on their visible or measurable characteristics. With the advent of biotechnology, the process has been facilitated by the use of marker-assisted selection (MAS) with several markers developed (Collard *et al*., 2007; Singh *et al*., 2014). Host plant resistance is the foundation of cereal insect pest and pathogen management programs and several *D. noxia* resistance (*Dn*) genes and leaf rust resistance (*Lr*) genes have been introduced into commercial cultivars (McIntosh *et al*., 2012; Khan *et al*., 2013). Although these genes confer resistance, new biotypes of the insect pest or races of the pathogen could bridge the current resistance thereby necessitating the continuous search for new sources of resistance (Terefe *et al*., 2011; Botha *et al*., 2014). Initially, the genetics of resistance was thought to follow the gene-for-gene hypothesis, but this has been found to be more complex with little known on the mechanism of resistance of these genes (Flor, 1971; Botha *et al*., 2005). From the protein perspective, few reports have documented proteins that are involved in pathogen-wheat or pest-wheat interactions (Rampitsch *et al*., 2006). Therefore, the identification and determination of the function of differentially expressed proteins in response to stress imposed by the leaf rust fungus and the phloem-feeding Russian wheat aphid would enhance our understanding of plant stress response pathways and therefore facilitate the development of new resistant crop varieties.

1.7.1 Hypothesis

Leaf rust infection and Russian wheat aphid infestation on resistant wheat cultivars result in similar stress responses that cause the differential regulation of various proteins within the plant.

1.7.2 Aim of the study

To identify the resistant mechanism(s) of wheat to leaf rust infection and Russian wheat aphid infestation and possible synergistic activation of stress response signalling pathways.

1.7.3 Objectives

The specific objectives of this study were to:

(i) determine the resistance mechanism of SST 347 and SST 356 when stressed with RWA SA1.
(ii) determine the resistance mechanism of SST 347 and SST 356 when stressed with leaf rust.
(iii) determine the synchronicity of leaf rust and aphid resistance mechanisms.

CHAPTER TWO

MECHANISM OF RUSSIAN WHEAT APHID RESISTANCE IN WHEAT CULTIVARS

2.1 Introduction

Russian wheat aphid (RWA) is one of the most devastating insect pests of wheat worldwide including South Africa (Botha *et al*., 2014). Plants protect themselves from biotic and abiotic stresses by developing a wide range of strategies termed ‘defence’ or ‘stress’ responses. In the course of a particular stress, a subset of genes are induced with some being early responsive and others late to cope with the impending stress (Giordanengo *et al*., 2010). Although both are necessary to defend the host against various cues, early response genes play a key role in perceiving and amplifying different stress signals and induction of further downstream gene expression. In the small grain production sector of South Africa, leaf rust and aphid-phloem feeding are sources of biotic stress in wheat plants causing significant yield losses (Terefe *et al*., 2014; Botha *et al*., 2014).

The application of systemic insecticides has successfully controlled RWA, however, due to the intermittent nature of the pest they remain costly and hazardous (Schotzko and Bosque-Perez, 2000). Consequently host plant resistance remains one of the most favorable control strategies for *Duiraphis noxia* as it is sustainable and does not increase costs of production (Schotzko and Bosque-Perez, 2000; Botha *et al*., 2014). Several resistant lines of *Triticum aestivum* (L) (Poaceace) have been developed over the years to control populations of *D. noxia* (Mordvilko) (Hemiptera: Aphididae) (Messina and Bloxham, 2004) but the mechanism of resistance remains largely unknown (de Wet and Botha, 2007). It has been debated that susceptible and resistant wheat cultivars react differently at the genomic and thus at the cellular levels through changed cellular function (de Wet and Botha, 2007). In South Africa, some of the commercial wheat cultivars released are resistant (R), moderately resistant (MR), moderately susceptible (MS) or susceptible (SS) to the prevailing aphid biotypes (ARC-SGI, 2015 production manual).

Genomic and trascriptomic studies have been conducted to explain structural components and molecular mechanism of resistance responses in wheat (Wang *et al*., 2010; Chen *et al*., 2013). Recently, the rapidly developing proteomic (systemic analysis of the protein component of a genome) technology is a very important tool to provide real insights into the extremely complex plant defence response to various stresses including pathogen/pest attacks (Agrawal *et al*., 2005). This technology allows the global analysis of gene products in various tissues and physiological states of cells (Patterson and Aebersold, 2003; Phizicky *et al*., 2003). The protein component of a cell is known to vary from cell to cell even under different stress conditions, therefore studying and analysing the proteome of individual cells would result in the identification of proteins actually present therein (Corton *et al*., 2004). Proteomics determines the presence, relative abundance and post-translational modification state of a large fraction of the proteins in a sample (Wilkins *et al*., 1996). Proteins are directly involved in cellular structure, regulation and metabolism and therefore proteomics can often yield a more informative and accurate picture of the state of a living cell than the analysis of the genome or transcriptome. Therefore, integrating data from genomics, transcriptomics and proteomics will allow for a more precise knowledge of how changes in gene expression lead to changes in metabolism.

Proteomics has long been associated with techniques such as two-dimensional gel electrophoresis (2-DE) and Liquid chromatography Mass spectrometry (MS) to profile proteins in biological samples (Schevchenko *et al.*, 1996; Wilkins *et al*., 1996; Nakamura & Oda, 2007). Classical 2-DE had limitations in resolution and reproducibility of gels and this has been overcomed by the use of immobilized pH gradient (IPG) strips (Gorg, 1991). The upgraded techniques in 2-DE permitted comprehensive protein visualisation on gels and proteomics was further advanced by the development of biological MS and the growth of searchable sequence databases (Park, 2004). The MS techniques that were developed for the ionization of proteins and peptides included matrix-assisted laser desorption ionisation (MALDI) and electrospray ionisation (ESI) (Karas and Hillenkamp, 1988; Fenn *et al*., 1989). MS combined with time of flight (TOF), ion trap and triple-quadrupole tandem MS (MS/MS) spectrometers offer high sensitivity and mass accuracy (Aebersold & Mann, 2003). Mass spectrometry has therefore become an invaluable tool in proteomics to identify differentially regulated proteins as well as quantitate proteins in high throughput systems (Domon & Aebersold, 2006; Elias *et al*., 2005). Although 2-DE-based proteomics has proven powerful for the global analysis of proteins, one of its greatest challenges is the reproducible separation of complex protein mixtures while retaining both qualitative and quantitative relationships. Although, other combinations of methods can be used to separate and analyse proteins, 2-DE is unique in its ability to separate hundreds to thousands of proteins simultaneously (Choe and Lee, 2003).

Two different electrophoretic separations are involved in the 2-DE technique; Isoelectric focusing and SDS-PAGE to separate proteins according to their isoelectric point (pI) and molecular weight respectively. Individual protein spots from gels could then be identified by mass spectrometry (MS) of their tryptic peptides. Other common methods of proteome analysis involve the proteolytic digestion of protein samples and the chromatographic separation of their resulting peptides coupled directly to mass spectrometry. The 2-DE is advantageous in that it maintains proteins in their intact states and enables the study of isoform distribution that is not possible if the samples are proteolytically digested prior to separation.

2.2 MATERIALS AND METHODS

2.2.1 Materials

Seeds of two wheat cultivars, SST 347 (R to RWA SA1 and to leaf rust race 3SA145) and SST 356 (MR to RWA SA1 and S to leaf rust race 3SA145) (Figure 2.1) were obtained from SENSAKO, Bethlehem while Russian wheat aphids biotype one (RWA SA1) clones were obtained from the Agricultural Research Council, Bethlehem South Africa. Hygromix (potting soil, river sand and vermiculite in the ratio 2:1:1) and hygrofert were purchased from Hygrotech, East London, South Africa. The total protein extraction, protein quantification (RCDC) kits, 2-DE clean-up kits, 2-DE starter kits; IPG strips, rehydration trays, criterion gels (14%), sypro ruby protein stain and PDQuest software were obtained from Bio-Rad (U.S.A.). Liquid nitrogen was obtained from the Department of Chemistry, Rhodes University (Grahamstown, South Africa).

Figure 2.1: SST 347 on the left and SST 356 the right (taken from Klein Karoo, South Africa; www.swwdmarketing.co.za).

2.2.2 Russian wheat aphid

Colonies of RWA SA1 were maintained on Scheepers using Pwlio (Coviron S1OH; Controlled Environments Limited, Winnipeg, Manitoba Canada; Analytical Scientific Instruments CC South Africa) in aphid cages **(**Figure 2.2**)** until used to stress plants. The Scheepers plants were replaced weekly to ensure succulent hosts for the aphids. The chamber was set at 24 ºC, 2 Lamps (0352 µMOL), CO_2 (ambient) and humidity of 60% with 16-h photoperiod. To maintain nutrient content, plants were watered twice a week with hygrofert (1g/L).

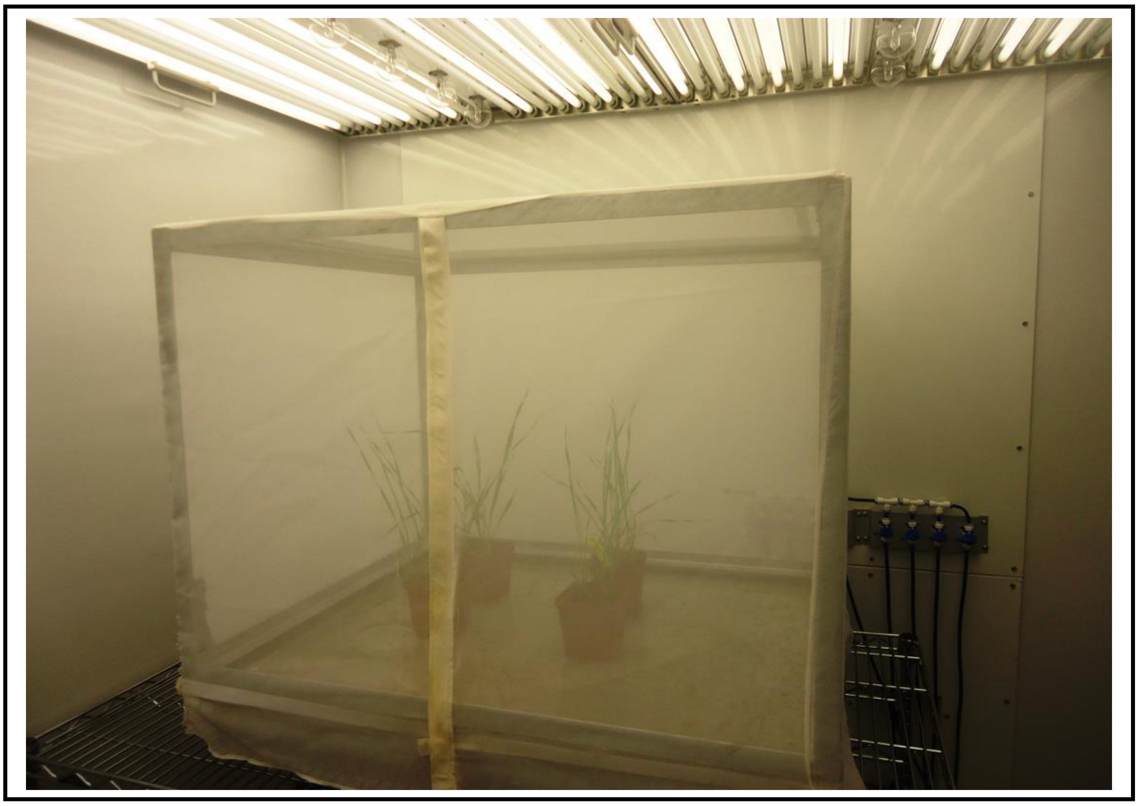


Figure 2.2: RWA cage in the Conviron

2.2.3 Experimental design

Seeds of SST 347 and SST 356 were germinated in sterile pots with hygromix soil and were placed in cages housed in the Pwlio Conviron growth chamber set at 24 ºC, 60% humidity, CO_2 (ambient), 2 lamps (0352 µMOL) and a day/night cycle of 16 hr/ 8 hr. A total of 372 plants were used which were grouped into four categories comprising of SST 347 (uninfested/control); SST 347 (Test/infested with aphids); SST 356 (uninfested/control); SST 356 (Test/infested with aphids) (Figure 2.3). Each group was made up of three pots with 3 plants per pot. The test and the control plants were placed in different cages to prevent contamination.

The plants were nourished twice a week by watering with hygrofert solution. Plants at 3-leaf stage of development (2 weeks old) were stressed with RWA SA1 with approximately ten aphids introduced to each plant for both cultivars. Control plants with no aphids were placed in a separate cage in the conviron to avoid contamination. Aphids were allowed to feed on the plants for nine days.

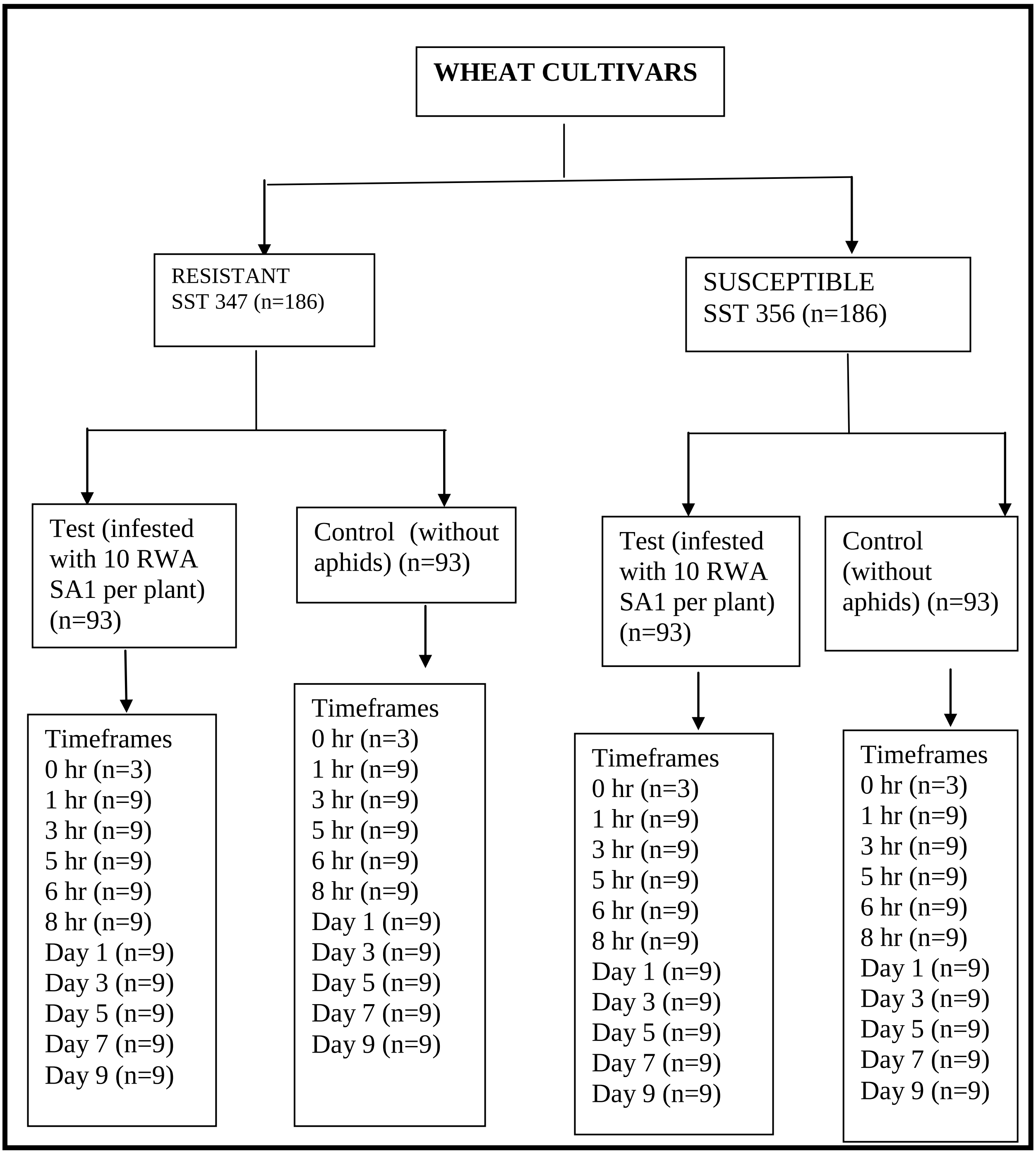


Figure 2.3: Schematic presentation of wheat cultivars used (n = number of plants) in the RWA experiment.

2.2.4 Leaf harvest

As the aphids were feeding on the plants, leaves were harvested at 0 (0 hrs), 1 hrs, 3 hrs, 5 hrs, 6 hrs, 8 hrs, 12 hrs, Day 1 (24 hrs), Day 3 (72 hrs), Day 5 (120 hrs), Day 7 (168 hrs) and Day 9 (216 hrs). Two leaves were cut from each of the three plants from three different pots per time frame using a pair of scissors. Control plants were harvested simultaneously with care taken not to contaminate the uninfested plants with aphids. The leaves from each pot were pooled into well labelled 15 mL sterile falcon tubes to eliminate any plant to plant variations that may occur. The leaves were snap-frozen in liquid nitrogen and stored in a -80 ºC freezer until further processing.

2.2.5 Determination of aphid population growth and physical responses (damage) on the cultivars during the experimental period

The aphid population growth on the two wheat cultivars was determined during the experimental period as previously described by Jimoh *et al*. (2011). Briefly, aphid population on leaves of each plant were assessed at 1-14 DAI. The adaxial and abaxial surfaces of the levels of every plant (whole plant) of both cultivars were carely examined and the numbers of live aphids were non-destructively counted with the aid of a hand lens. Visible manifestation of aphid feeding related damage was simultaneously assessed by using the scoring system previously described by Jimol *et al*. (2011) as explained in Table 2.1. Population data obtained were statistically analysed using ANOVA (Analysis of Variance) to determine significant differences between the means of each group (Jimor *et al*., 2011).

Table 2.1: Russian wheat aphid damage symptoms used for scoring (Jimol *et al*., 2011)

Scale	**Description**
Chlorosis[1]	
0	Plant appears healthy, no chlorotic or necrotic spot(s) on any leaf.
1	Plant appears healthy, may have few isolated chlorotic or necrotic spot(s).
2	Chlorotic spots become more noticeable, up to 5% of total leaf area.
3	Chlorotic spots are larger and more numerous, up to 15% of total leaf area.
4	Chlorosis covers up to 25% of the total leaf area. Some streaking may become apparent, especially along the midrib.
5	Chlorotic spots may begin to coalesce or definite streaking may occur. Chlorosis covers up to 40% of the total leaf area.
6	Larger chlorotic areas form coalesced spots, leaves start to die back from tips. Chlorosis covers up to 55% of the total leaf area.
7	Further symptom development; chlorosis covers up to 70% of the total leaf area.
8	Extensive chlorosis and necrosis; up to 85% of the total leaf area affected.
9	Plant death or no recovery possible.
B leaf roll[2]	
1	Leaves are flat, no apparent rolling.
2	Leaves are folded and/or loosely rolled at the margins.
3	Tightly or completely rolled leaves.

[1] Chlorosis scale adapted from Webster *et al*. (1987).
[2] Leaf rolling scale adapted from Burd *et al*. (1993).

2.2.6 Total protein extraction

Total protein was extracted from the harvested leaves using the ReadyPrep™ Protein extraction kit (total protein) (Bio-Rad, US) following the manufacturer's instructions. In summary, the harvested leaves were homogenised under liquid nitrogen using sterile mortar and pestle into a fine powder and 0.1 g quickly transferred into a sterile 1.5 mL microcentrifuge tubes. Two hundred microliters of freshly prepared extraction buffer 1 (7 M urea, 2 M thiourea, 1% (w/v) ASB-14 detergent, 40 mM Tris base and 0.001% bromophenol blue) containing TBP reducing agent (200 mM tributylphosphine in 1-methyl-2-pyrrolidinone) was added to the tubes. Samples were placed on ice and the suspensions sonicated with an ultrasonic probe to disrupt the cells and fragment genomic DNA. Sonication was done using 30 secs bursts 3 times with the suspensions chilled briefly on ice between each burst. Tubes were centrifuged using a benchtop centrifuge (Hermle Z33, Lesec) at maximum speed (21500 x g) for 20 minutes at room temperature to pellet the debris. The supernatants were transferred into clean sterile 1.5 mL microcentrifuge tubes and the pellets discarded. Samples were stored in aliquots at -80 ºC until further analysis.

2.2.7 Protein quantification

The RC DC Protein Assay Kit from Biorad (US) was used to determine the concentration of protein in the samples after extraction following the manufacturer's instructions. The Microfuge Tube Assay Protocol (1.5 mL) was used as described. Briefly, Bovine serum albumin (BSA) stock (2 mg/mL) was prepared by dissolving 0.002 g of the powder in 1mL of distilled water and various dilutions made for the standard curve. A 10 fold serial dilution of the samples was made. Twenty-five microliters of standards and each dilution were pipetted into clean, dry 1.5 mL microcentrifuge tubes. One hundred and twenty-five microliters of RC reagent I added to each tube and the tubes vortexed and incubated at room temperature for 1 minute. After the incubation, 125 µL of RC reagent II was added to each tube and the tubes vortexed briefly and centrifuged at 21500 x g for 3 minutes. The supernatant was discarded by inverting the tubes on clean, absorbent tissue paper and the liquid allowed to drain completely from the tubes. Reagent A^1 (127 µL) prepared by adding 5 µL reagent S to 250 µL of reagent A was pipetted to each microcentrifuge tube, vortexed and incubated at room temperature for 5 minutes. The tubes were again vortexed and 30 µL of the re-suspended protein in A^1 was pipetted into three wells (triplicates) of the microtiter plate and 237 µL of DC Reagent B added to each well. The absorbance was read at 750 nm after 15 minutes of incubation in the plate reader (SynergyMix, Biotek). BSA standard curves were created using Microsoft Excel® and the protein concentration of samples interpolated.

2.2.8 Protein clean up

Protein extracts were cleaned using ReadyPrep™ 2-D Clean up Kit (Biorad, USA). This kit serves to prepare low conductivity samples making them suitable for Isoelectric focusing (IEF) and 2-DE. It also concentrates proteins to improve spot detection (Bio-Rad, 2014 manual). Proteins (1-500 µg) in a final volume of 100 µL of 2-D starter Kit Rehydration/Sample buffer were transferred to 1.5 mL microcentrifuge tubes. Three hundred microliters of precipitating reagent I was added to the protein sample and mixed well by vortexing. The mixture was then incubated on ice for 15 minutes followed by the addition of 300 µL of precipitating reagent II. The tubes were mixed thoroughly by vortexing and centrifuged at maximum speed (21500 x g) for 5 minutes to form tight pellets. Tubes were removed promptly to avoid dispersing of pellets and the supernatants discarded by use of pipette. The tubes were positioned in the centrifuge as before and centrifuged for 30 seconds to collect any residual liquid that was eventually discarded with the aid of a pipette. Forty microliters of wash reagent 1 was added onto the pellet and the tubes positioned in the centrifuge as before, centrifuged at maximum speed (21500 x g) for 5 minutes and wash reagent discarded. Thereafter, 25 µL of ReadyPrep proteomic grade water was added on top of the pellet and the tubes vortexed for 20 seconds. This was followed by the addition of 1mL of wash reagent 2 (prechilled at -20 ^{o}C for at least 1 hr), and 5 µL of wash 2 additive. The tubes were vortexed for 1 minute and incubated at -20 ^{o}C for 30 minutes during which the tubes were vortexed for 30 seconds every 10minutes. After the incubation period, the tubes were centrifuged at top speed for 5 minutes to form tight pellets and the supernatants discarded. The tubes were again centrifuged briefly for 30 seconds and any remaining wash removed. The pellets which appeared white at this stage were air dried at room temperature for 5minutes; when sufficiently dried it looked translucent. Each pellet was re-suspended by adding 250 µL of 2-DE sample/rehydration buffer [8 M urea, 2% CHAPS, 50 mM dithiothreitol (DTT), 0.2% (w/v) Bio-Lyte®, 3/20 ampholyte and bromophenol blue (trace)]. To clarify the protein samples, the tubes were centrifuged at maximum speed for 3 minutes. The protein samples were quantified using the RCDC Protein Assay Kit (Biorad, USA) as described above (section 2.2.6). The supernatant was then used immediately to rehydrate IPG strips or stored at -80 ^{o}C for later analysis.

2.2.9 Rehydration of IPG strips

IPG strips were rehydrated in Zoom® IPGRunnerO Cassettes prior to performing IEF. Broad-range pH 3-10 NL (11 cm) ReadyStrip^{TM} IPG strips were rehydrated overnight with 200 µg of protein sample in a final volume of 185 µL 2-D rehydration/sample buffer following the manufacturer’s instructions; 185 µL of diluted protein samples were pipetted each as a line along the back edge of respective channels of the rehydration/equilibration tray. Using forceps, the coversheet of the strips were pealed off and strips placed gently gel side down onto protein samples avoiding air bubbles being trapped. The strips were overlaid with 2 mL of mineral oil to prevent evaporation during the rehydration process. The tray was covered and left to rehydrate on a level bench top overnight (11-16 hrs). Samples were run in duplicates to eliminate pot to pot variations that may occur.

2.2.10 Isoelectric focusing (IEF)

A clean dry PROTEAN IEF focusing tray (same size as the rehydrated IPG strips) was placed on the bench top and paper wicks that would fit over the electrodes were placed with the help of a forcep at both ends of the channels covering the wire electrodes. Channels with same numbers as those used during rehydration were used. Eight microliters of nanopure water was pipetted onto each wick to wet it. With a forcep, the rehydrated IPG strips were removed from the rehydration tray and held vertically for 8 seconds to allow the mineral oil to drain, and then the IPG strips were transferred to the corresponding channel in the focusing tray with gel side maintained downward. Each IPG strip was overlaid with 2 mL of fresh mineral oil and checked for air bubbles beneath the strips. The IEF focusing tray was covered with the positive “+” side of the lid to the left. The tray was then placed into the IEF cell programmed as described on Table 2.2 using default temperature of 20 ^{o}C with a maximum current of 50 µA/strip.

Table 2.2: IEF Program for 11cm (pH 3-10)

11cm	**Voltage**	**Time/V-hrs**	**Ramp**
Step 1	250	20 min	Rapid
Step 2	8000	1 hr	Linear
Step 3	8000	26,000 v-hr	Rapid
Step 4	1500	∞	Hold

On completion of the electrophoresis run, strips were removed from the focusing tray and transfered into a clean dry tray that matches the length of the IPG strips. The strips were held vertically for 5 seconds with forceps to allow the mineral oil to drain before placing them in the new tray with gel side facing up. When SDS PAGE was not performed immediately, the tray was wrapped in plastic and stored at -80 °C.

2.2.11 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Prior to separation of proteins by SDS-PAGE, the IPG strips were equilibrated in two buffers, equilibration buffer I [20 mL of 6 M urea, 2% SDS, 0.375 M Tris-Hcl (pH 8.8), 20% glycerol, and 2% (w/v) DTT] and II [20 mL of 6M urea, 2% SDS, 0.375 M Tris-Hcl (pH 8.8), 20% glycerol]. Four milliliters of equilibration buffer I was added to each channel of the rehydration tray containing an IPG strip with gel side facing upward and the tray placed on an orbital shaker and gently shaken at a slow shaker speed for 10 minutes. At the end of the 10 minutes incubation, the used equilibration buffer I was carefully decanted and the trays flicked a couple of times to remove the last few drops of equilibration buffer I. Four milliters of equilibration buffer II (containing iodoacetamide) was added to each strip and the tray returned to the orbital shaker for 10 minutes with the equilibration buffer II decanted at the end of the incubation. A 100 mL graduated cylinder or a tube with the same length or longer than the IPG strip length was filled with 1X Tris-glycine-SDS running buffer and bubbles on the surface of the buffer removed with a Pasteur pipette. The precast gels (Criterion^{TM}TGX^{TM}, 4-20%, Biorad, USA) were prepared by removing the gels from their packs, the gel tops rinsed twice with distilled water and excess water remaining in the IPG well blotted out with Whatman 3 MM or similar blotting paper. The gels were placed on the bench with the top of the gel facing upward and the back (tall) plate on the bottom. The IPG strips were removed from the disposable rehydration/equilibration tray and dipped briefly into the graduated cylinder containing 1X Tris-glycine-SDS running buffer. The strips were placed gel side up onto the back of the plate of the Criterion gel above the IPG well. The gels with the IPG strips resting on the back plate were positioned vertically on a test tube rack with the short plate facing upward. The overlay agarose solution was pipetted into the IPG well of the gel and with the aid of forceps, the strip carefully pushed into the well with care taken not to trap any air bubbles beneath. The gels were then allowed to stand vertically in the test gel rack for 5 minutes to allow the agarose to solidify before proceeding. The gels were mounted into the gel box. The reservoirs were filled with 1X Tris-glycine-SDS running buffer and electrophoresis carried out in a Bio-RAD Mini Protein II System at a constant voltage of 200 V for 35 minutes. The migration of the bromophenol blue present in the overlay agarose solution was used to monitor the progress of the run.

2.2.12 Staining of gels

After electrophoresis, the gels were removed from the gel cassette, placed in a clean plastic container and fixed for 30 minutes in a mixture of 10% methanol and 7% acetic acid. After fixation, the gels were washed 3 times in distilled water for 10 minutes each. The wash solution was removed and the gels covered with 50 mL SYPRO Ruby protein gel stain and wrapped in aluminium foil. Gels were stained continuously with gentle agitation on a shaker overnight (16-18 hrs). Destaining was done in the fixing solution for 60 minutes to decrease background fluorescence. The gels were again washed in water, visualised and photographed using Alliance 4.7 Transilluminator (UVITEC Ltd, Cambridge, UK).

2.2.13 PDQuest analysis of gels

The PDQuest 2-DE Analysis Software Version 8.0 (Biorad) was used to study the duplicate gels in order to determine the number of visible spots as well as the spot variance between gels following the manufacturer’s instructions. Gels were grouped and analysed using the Spot Detection Parameter Wizard with a sensitivity of 0.49 while allowance was made for horizontal and vertical streaks removal. The gel software analysis package relied on manual “calibration” through the identification of a faint spot, a large spot and the largest spot cluster, allowing for some user input in terms of parameters. A speckle filter set at 50 was applied in order to remove speckles. A Gaussian statistical model was used to detect and fit spots while a local regression model was selected for data normalisation.

2.2.14 Protein analysis and identification

2.2.14.1 Re-staining of gels

Gels with protein spots of interest were stained with colloidal coomassie blue G250 (Resyn Biosciences) following the manufacturer's instructions to enhance visibility of spots for cutting. Briefly, the gels were placed in clean staining dishes and covered with the staining solutions. These were placed on an orbital shaker (Ultra Rocker, Biorad) set at a low speed (20 RPM) for 1-2 hours. According to the assay, protein spots may be visible within 5-10 minutes and reach a maximum intensity in 1 hour. After staining, the gels were rinsed in a large volume of mass spectrometry grade water, 3 times for 10-15 minutes each until the background was transparent.

2.2.14.2 Excision of protein spots

Formic acid (FA), Acetonitrile (ACN), Ammonium bicarbonate (NH_4HCO_3), Dithiothreitol (DTT), Iodoacetamide, Calcium Chloride ($CaCl_2$), Methanol (MeOH) and Trypsin were used (appendix I) with volumes dependent on the number of samples to be analysed. The entire process was performed at the Center for Scientific and Industrial Research (CSIR) Pretoria. Protein spots of interest were excised as described by Shevchenko *et al.* (2007) with slight modifications. Special care was taken during the entire process to prevent massive keratin contamination of the samples. The gels were placed on the sample tray (white background) of the Pharos Fx^{TM} plus molecular Imager (Biorad, USA) and differentially regulated protein spots excised with a clean sterile surgical blade. The gel slices were diced into smaller pieces (1x1 mm) into well labelled 0.5 mL Protein Lo-Bind eppendorf tubes and destained as follows. Two hundred microliters of 50 mM NH_4HCO_3/ MeOH (1:1, vol/vol) was added to each tube and vortexed in a Thermomixer comfort (Eppendorf) for 20 minutes. The supernatants were carefully discarded not to pick the smaller gel pieces. This step was repeated to ensure that the gel slices were transluscent. Followed was the addition of 100 µL of 75% ACN and vortexed in a thermomixer for 20 minutes. The acetonitrile was removed and gel pieces speed vac in a Vacuum dryer (CentriVap Concentrator: LABCONCO) for 10 minutes to complete dryness.

2.2.14.3 Reduction and alkylation

The gel pieces were covered with 25 µL of 10 mM DTT in 25 mM NH_4HCO_3, the tubes were vortexed and spun briefly. Tubes were incubated at 60 °C for 1hour in the thermomixer thereafter chilled to room temperature (22-23 °C) and 250 µL of ACN added. The tubes were incubated at room temperature for 10 minutes. The supernatants were discarded and 25 µL of 55 mM iodoacetamide in 25 mM NH_4HCO_3 added to the gel pieces. The reaction was allowed to proceed in the dark for 20 minutes at room temperature (22-23 °C). The supernatants were discarded and the gels washed by adding 100 µL of 25 mM NH_4HCO_3 in milliQ-H_2O and tubes vortexed for 10 minutes. The supernatants were again discarded and the gels dehydrated by adding 100 µL of 25 mM NH_4HCO_3 in ACN. The tubes were vortexed for 5 minutes and spun for another 5 minutes after which the supernatants were discarded. The dehydration step was repeated and the gel pieces speed vac for 20 minutes to completely dry the gels.

2.2.14.4 Protein digestion

Several enzymes can be used to cleave proteins including trypsin, LysC endoprotease and pepsin (Ren *et al.*, 2009). In this study proteins were digested with trypsin that cleaves on the C-terminus of the lysine and arginine residues. Its ability to produce smaller average lengths as well as charge retaining residues at the C-terminus of the tryptic peptide makes the tryptic peptides better fit for modern reverse phase liquid chromatography and tandem mass spectrometric analysis. Gel pieces were rehydrated on ice in enough trypsin digestion solution. Ten microliters of freshly prepared trypsin solution [180 µL of 25 mM NH_4HCO_3 added to the trypsin aliquotes (10 ng/µL stock)] was added to the protein plugs and incubated on ice for 30 minutes. After the incubation, the volume was topped up with 25 µL of 25 mM Na_2HCO_3 and the gel pieces rehydrated on ice for further 90 minutes. A quick spin was performed in a Hermle Z100M centrifuge and the tubes incubated overnight at 37 °C in a water bath (Memmert).

2.2.14.5 Peptides Extraction

After the overnight in-gel trypsin digestion of the protein samples, the digest solutions (aqueous solution) were transferred into clean 0.5 mL Protein Lo-Bind tubes. To extract any remaining peptides the following procedure was performed. To the gel pieces, 30 µL of 50% ACN in 5% formic acid was added. The tubes were vortexed for 25 minutes in a thermomixer, briefly spun and sonicated (Bandelin Sonorex) for 5 minutes. The aqueous extraction transferred to the tubes containing peptides from the overnight digestion and the procedure was repeated to ensure extraction of all peptides. The total aqueous extractions were dried in a speed vac and the digests stored at -20 °C until MS/MS.

2.2.14.6 Identification of peptides

Digests were resuspended in 20 µL, 2% acetonitrile/0.2% formic acid and analysed using a Dionex Ultimate 3000 RSLC system coupled to an AB Sciex 6600 TipleTOF mass spectrometer. Peptides were first de-salted on an Acclaim PepMap C18 trap column (100 µm × 2 cm) for 2 min at 15 µL/min using 2% acetonitrile/0.2% formic acid, than separated on Acclain PepMap C18 RSLC column (300 µm × 15 cm, 2 µm particle size). Peptide elution was achieved using a flow-rate of 8 µL/min with a gradient: 4-60% B in 15 min (A: 0.1% formic acid; B: 80% acetonitrile/0.1% formic acid). An electrospray voltage of 5.5 kV was applied to the emitter. The 6600 TipleTOF mass spectrometer was operated in Data Dependant Acquisition mode. Precursor MS scans were acquired from m/z 400-1500 using an accumulation time of 250 ms followed by 30 MSMS scans, acquired from m/z 100-1800 at 100 ms each, for a total scan time of 3.3 sec. Multiply charge ions (2+ - 5+, 400 -1500 m/z) were automatically fragmented in Q2 collision cells using nitrogen as the collision gas. Collision energies were chosen automatically as function of m/z and charge. Protein pilot v5 using Paragon search engine (AB Sciex) was used for comparison of the obtained MS/MS spectra with a custom database containing sequences of *Puccinia triticina* (Uniprot Swissprot), *Triticum aestivum* (Uniprot TrEMBL) and Russian wheat aphid (Uniprot TrEMBL) as well as a list of sequences from common contaminating proteins. Proteins with threshold above ≥ 99.9% confidence were reported.

2.3 RESULTS AND DISCUSSION

2.3.1 Wheat plant growth

The growth of wheat cultivars in the conviron is as shown, SST 347 (Figure 2.4) and SST 356 (Figure 2.5).

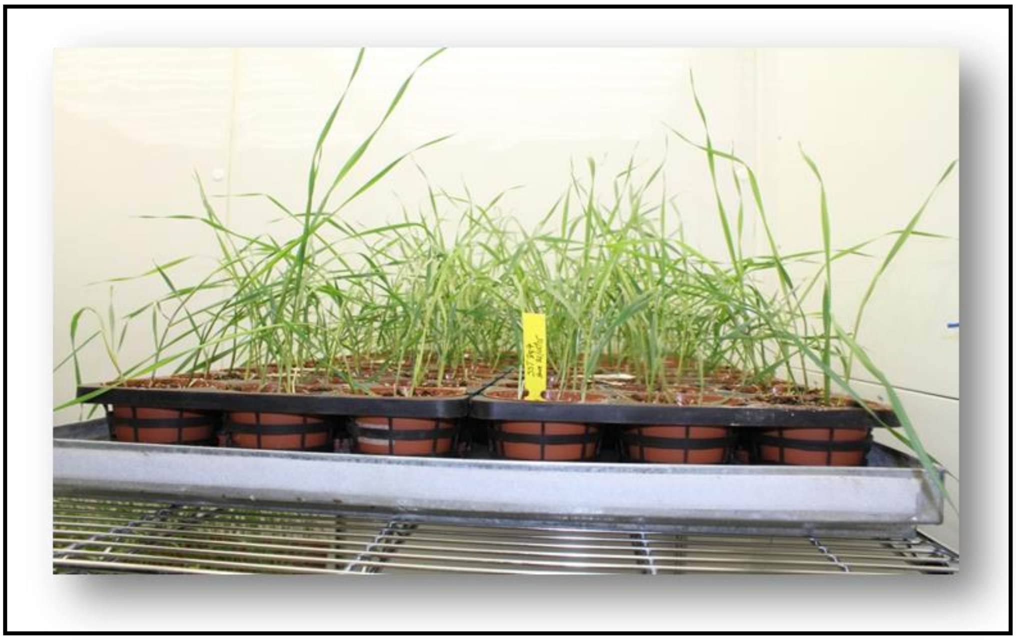


Figure 2.4: SST 347 wheat cultivar at three leaf stage

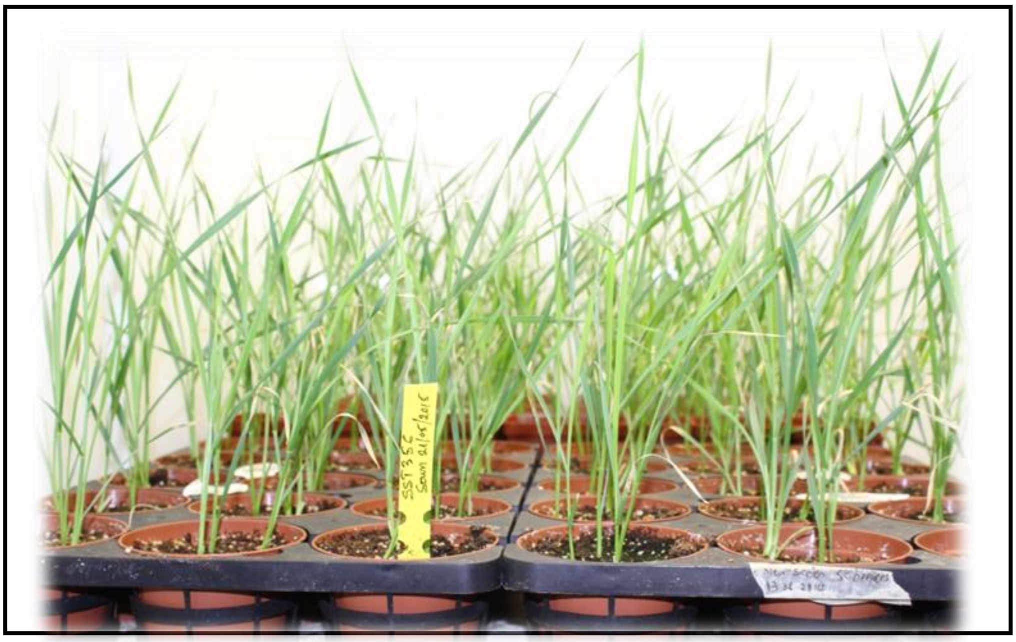


Figure 2.5: SST 356 wheat cultivar at three leaf stage

2.3.2 Physical responses (Damage) on the cultivars

The feeding activity of RWA SA1 resulted in visible damage to the leaves of moderately-resistant wheat cultivar SST 356, but was less evident in the resistant cultivar SST 347 (Figure 2.6). Symptoms such as chlorosis, necrosis, longitudinal yellow streak and leaf rolling were observed earlier (day 3 post infestation) as the aphids fed and reproduced on the moderately-resistant wheat cultivar (scale 3; Table 2.4), while the resistant cultivar only showed a few chlorotic and necrotic spots much later at day 7 post infestation (scale 1; Table 2.4). This result is inline with the Agricultural Research Council production manual (2014) which stated that SST 347 cultivar is resistant to Russian wheat aphid biotype 1 while SST 356 cultivar is moderately resistant.

Figure 2.6: RWA damage symptoms on SST 347 (A) and SST 356 (B)

2.3.3 Optimisation of protein extraction

Approxiately 0.1 g of ground wheat leaves were extracted with different protein extraction buffer volumes (200 µL, 300 µL and 400 µL). The 300 µL extraction buffer gave the highest protein concentration with the 200 µL volume giving a similar concentration (Figure 2.7). The 400 µL gave the lowest yield. The 200 µL was therefore chosen as the volume of extraction buffer to be used for all the protein extraction in this study.

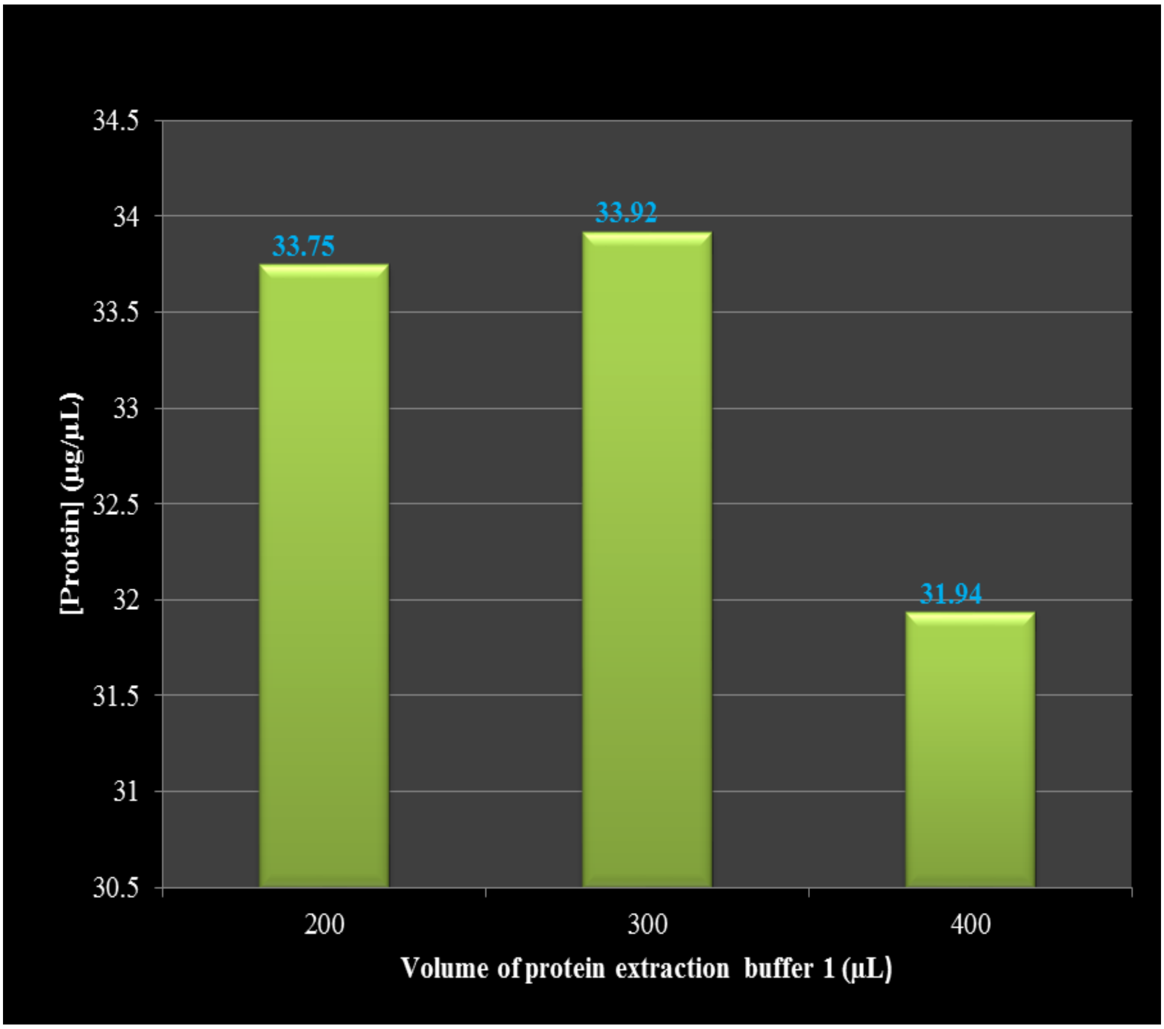


Figure 2.7: A histogram showing different concentrations of protein with their respective protein extraction buffer1 volumes.

2.3.4 Protein quantification

To determine the protein quantity obtained from extraction and after 2-DE clean up, a protein standard curve was generated using RCDC protein assay kit (Figure 2.8).

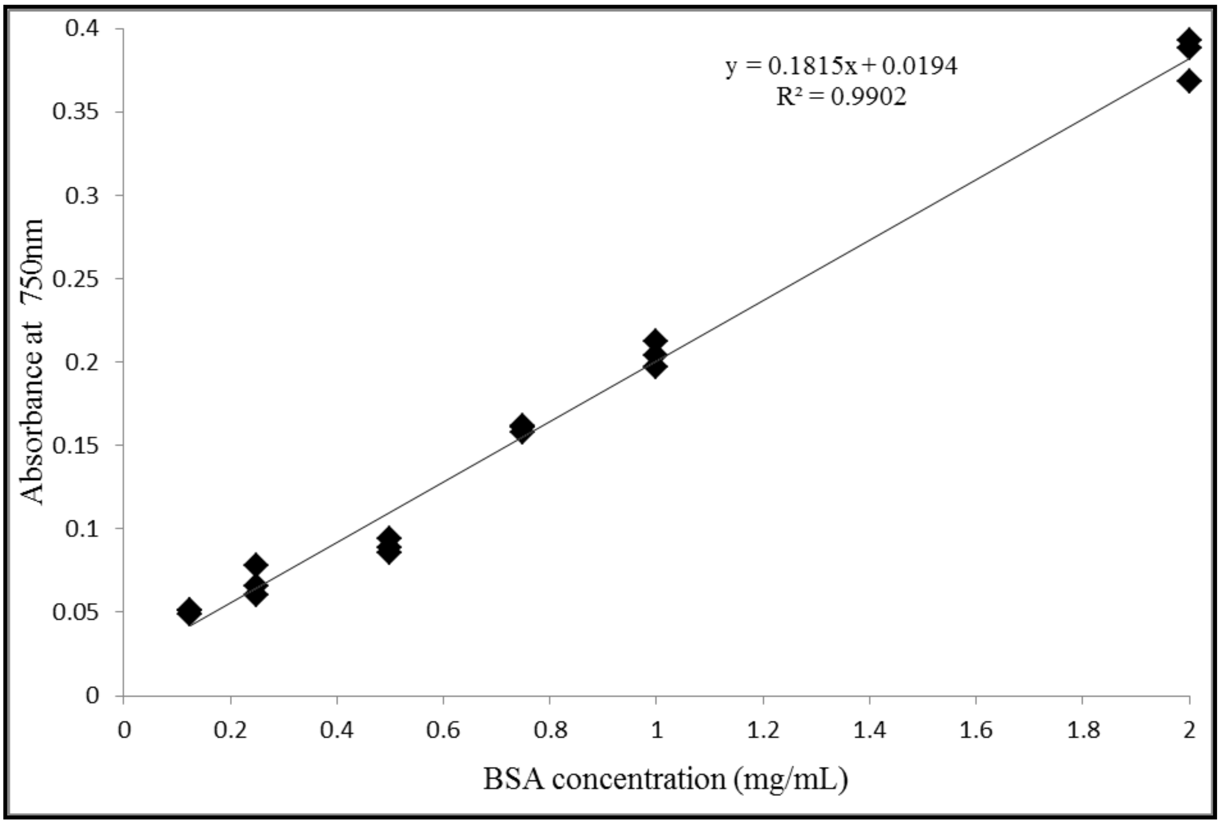


Figure 2.8: Protein standard curve generated using the RCDC protein assay kit (Bio-Rad, USA) with bovine serum albumin protein concentrations measuring from 0.125 mg/mL to 2 mg/mL. All concentrations were prepared in triplicates

The ReadyPrep protein extraction Kit (Total Protein, Bio Rad, U.S.A) is a simple, rapid and reproducible method to prepare total cellular protein extracts for 2-D gel analysis. The procedure can easily be scaled up or down to accommodate large/smaller amounts of cells or tissue with the entire procedure completed in about 45 minutes. One of the key reagents in the kit is the extraction buffer 1 with 2-3 mL of the buffer recommended per gram of plant tissue. According to the manufacturer's instructions, insufficient volume of the extraction buffer 1 may result in poor cell lysis and incomplete solubilisation of all protein types that may affect yield and purity. The volume of sample buffer that would give the best possible yield for the purpose of extraction was determined by assaying the protein obtained from three different extraction volumes (Figure 2.7). The 200 µL and 300 µL sample buffer volume yielded similar protein concentrations and therefore for economical reasons, the 200 µL volume per 0.1 g leaf material was chosen for subsequent extractions.

The chaotropic reagents (urea and thiourea) in the extraction kit help in achieving protein solubilisation by unfolding the protein via hydrogen bonds and electrostatic interactions (Speers *et al*., 2007). Although high concentrations of urea could interfere with trypsin digestion, their concentrations were kept within acceptable limits. The added ampholytes help to reduce protein aggregation by preventing proteins from interacting ionically with each other.

Protein extraction and sample clean up are the two most important steps in ensuring optimal resolution and reduce variability of 2-D gels with 2-D PAGE success depending on sample purity. The protein concentrations ranged from 16.80 mg/mL to 34.94 mg/mL after extraction. These results were three times higher than that of Mundondo (2015) who reported a protein concentration of 5.58 mg/mL to 10.83 mg/mL after protein extraction from Tugela *Dn* wheat leaves. After 2-DE clean up, results obtained showed a reduction in protein concentration, which ranged between 1.37 and 4 mg/mL indicating a huge loss in protein. These results are in line with those obtained by Louw, (2007) and Mundondo, (2015) who also reported a massive reduction in protein concentration after 2-DE clean up. This loss could be attributed to the removal of substances that interfered with reagents of the quantitation assays before the clean up resulting in high protein concentration (false high readings) before the clean-up process. The 2-DE clean-up procedure according to Bio-rad instruction manual works by quantitatively precipitating and concentrating proteins in a sample while leaving behind and washing away interfering substances such as ionic detergents, salts, nucleic acids, lipids and plant derived phenolic compounds all of which could negatively impact 2-DE. It is important to note that these protein reduction or loss occurred throughout indicating that the clean-up kit removes interfering substances.

2.3.5 Optimisation of staining

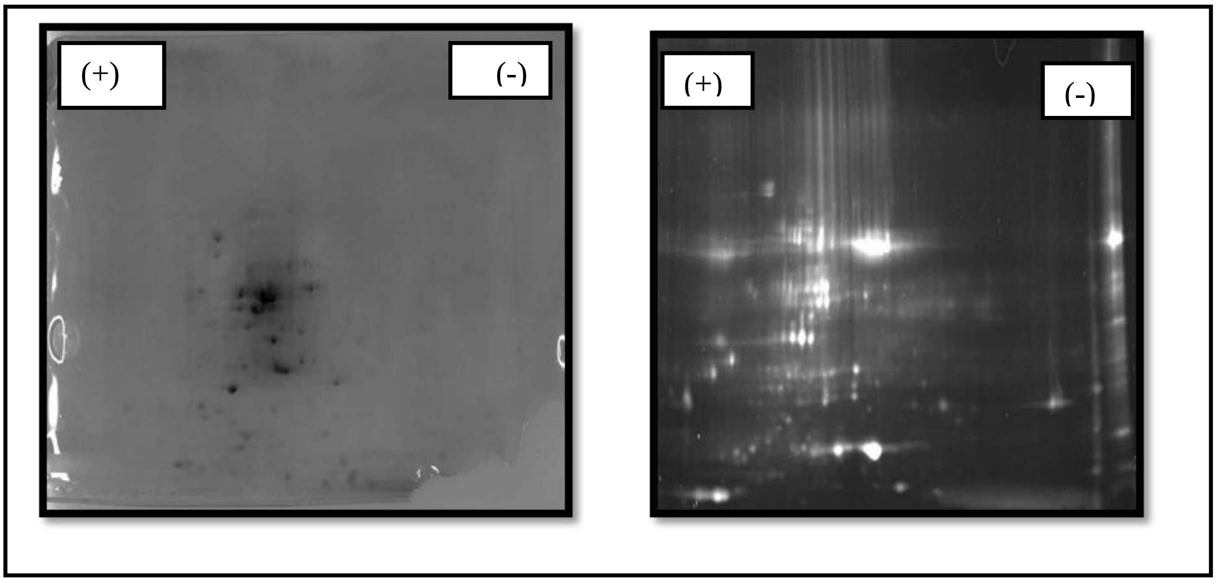


Figure 2.9: 2-DE gels showing protein spots in SST 347 wheat cultivar in response to aphid stress (left: Gel stain with Commasie Brilliant Blue and right: gel stained with Sypro ruby fluorescent gel stain).

The choice of a suitable stain is crucial for the success of 2-DE experiments. Ideally, a 2-DE stain must allow for qualitative analysis while simultaneously being sensitive enough for visualisation of low abundance proteins. Futhermore, a successful stain should possess a wide linearity and dynamic range while being affordable to the researcher (Westermeier and Naven, 2002). In this experiment, two stains were used to visualise the 2-DE gels in order to determine which stain would allow for the detection of a maximum number of spots under the given running conditions. The stains investigated were: Coomassie Brilliant Blue (40% methanol, 0.7% glacial acetic acid, 0.075% Coomassie brilliant blue R250) and Sypro Ruby gel stain (Biorad, USA). More spots (>20) were obtained from the gel stained with the latter (Figure 2.9).

According to literature, different stains interact differently with different proteins and for 2-DE analysis sensitive stains such as silver or fluorescent stains have been recommended (Corthals *et al.*, 2000). This therefore necessitates the optimisation of staining procedure for proteins from the wheat leaf tissue. Two stains were used for the optimisation process, they included Coomasie Brilliant blue (R250) and SYPRO Ruby fluorescent gel stain. From the gel picture (Figure 2.9) fewer protein spots were detected with coomasie an indication that coomasie brilliant blue appears to stain the broadest spectrum of protein spots which has also been reported by Carrol *et al*. (2000). On the other hand, more protein spots were observed on the gel stained with SYPRO Ruby gel stain making it more sensitive than Coomasie. Although Coomasie is a less expensive staining method, it lacks the sensitivity of fluorescent stains. This result corroborates that of Louw (2007) who also reported only a few spots on gels stained with Coomasie Blue-R-250. Protein detection and quantification commonly utilise the ability of dyes to bind non-specifically with an accompanying change in the spectral properties of the bound dye. Fluorescent dyes with this ability are particularly useful for protein analysis due to their high detection sensitivity thus making SYPRO ruby currently the most commonly used fluorescent protein stain. It is an endpoint stain with little background staining (High signal-to-noise characteristics) and it is widely used in conjunction with proteolytic digestion of excised protein spots and MS analysis (Lopez *et al*., 2000). Coomasie Blue R-250 typically has a detection limit of approximately 40 ng while Sypro ruby, regularly used in 2-DE application in studying low proteins, is sensitive to 1-10ng (Patton, 2000; Westermeier and Naven, 2002). SYPRO ruby gel stain detects low molecular weight proteins, is compatibility with high-throughput protocols and downstream analysis (MS and Edman sequencing), and detects glycoproteins, lipoproteins and metallo proteins that are not stained well by other stains (Patton, 2000). It also has a large dynamic linear range allowing quantitation of the protein spots across broad concentration range. Although, Coomassie Brilliant blue-R-250 is a less expensive staining method, it has numerous limitations including; some protein spots destain faster than the gel background during destaining which adds to a loss of protein spots and the linear range is limited in comparison to SYPRO ruby (Neuhoff *et al*., 1988).

The wheat leaf proteins were successfully isolated as protein spots were found on gels (Figure 2.9). The position of spots on the gels led to the general conclusion that negatively charged proteins (at the positive end) were mostly resolved. Most of the proteins resolved were in the relatively low molecular weight region of approximately 17 KDa to 55 KDa although a protein molecular weight marker was not used. However, this result is in line with that of Mak *et al.* (2006) who reported that relatively few high molecular weight proteins (>100 KDa) are present in wheat. The SwissProt database also showed that majority of the proteins were less than 50 KDa similar to what was reported by Louw (2007). In all gels, there was an outstanding band that was large in size and highly fluoresced and it was noticed to occupy almost the same position. This was thought to be RuBisco (ribulose bisphosphate carboxylase oxygenase), the most abundant protein in plants. This was also observed by Louw (2007) in whose gels were a very prominent band occurring at approximately 55 KDa. It was observed that the distribution of protein spots in the gels were similar, a probable indication that the protein content of wheat leaves was similar as recounted by Zhenhu and Jiangyan (2013).

2.3.6 PDQuest Analysis

Proteome maps of wheat leaf tissues from each treatment (a resistant and moderately resistant line) were compared using the PDQuest version 8.0 software and differentially regulated proteins identified by MS and MS/MS searches of the NCBI/Swiss-Prot and a locally restricted wheat EST database.

PDQuest analysis requires manual input of parameters such as selection of faint and large spots cluster (Figure 2.10A). The software also allowed the comparison of the two groups of gels (infested vs uninfested) with the master gel to determine matched and unmatched spots (Figure 2.10B).

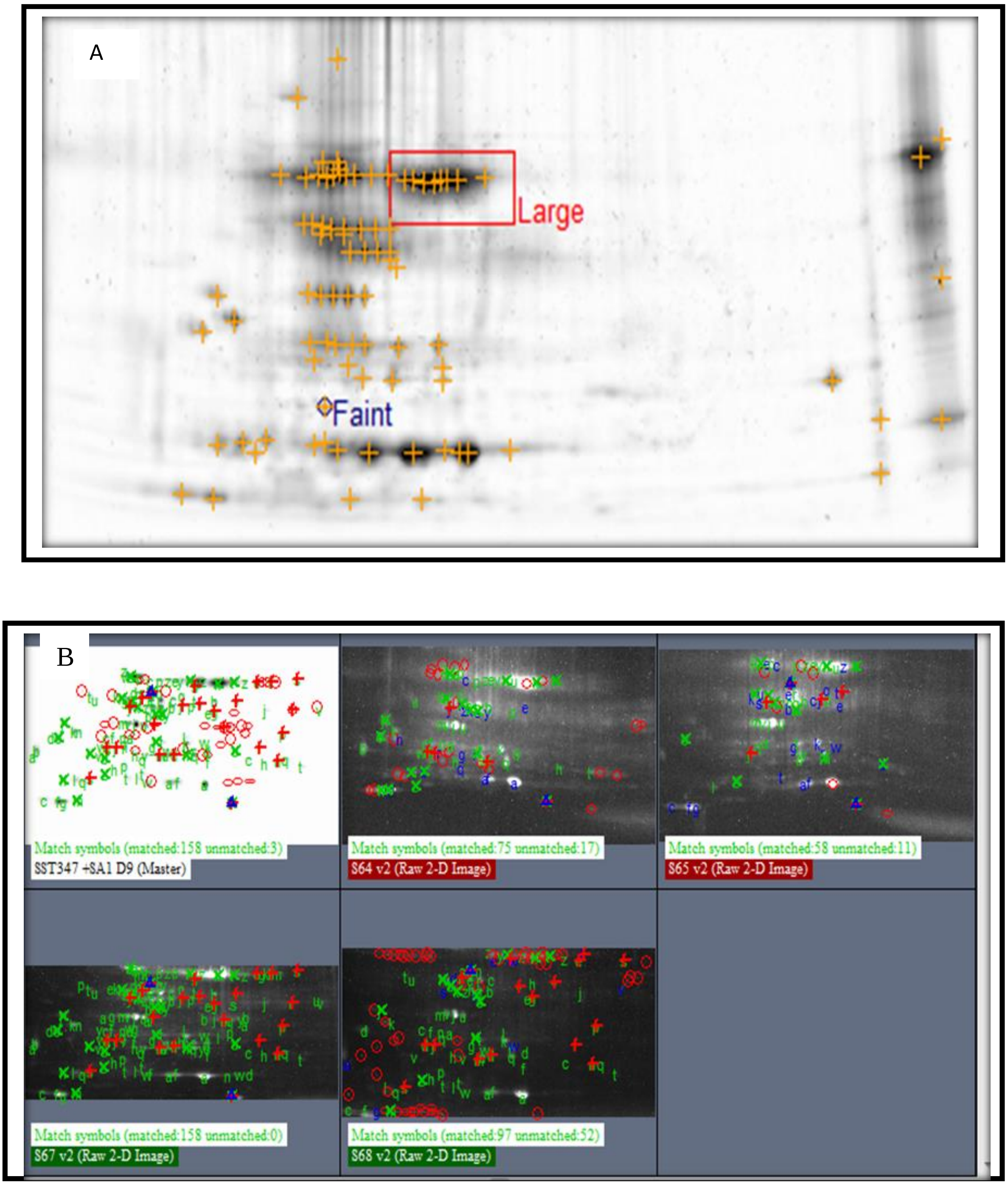


Figure 2.10 B: PDQuest^{TM} Basic Software (Bio-Rad) showing two steps taken during the analysis of the 2-DE gel samples showing differential expressed proteins in SST 347 wheat cultivar in response to RWA SA1 infestation.

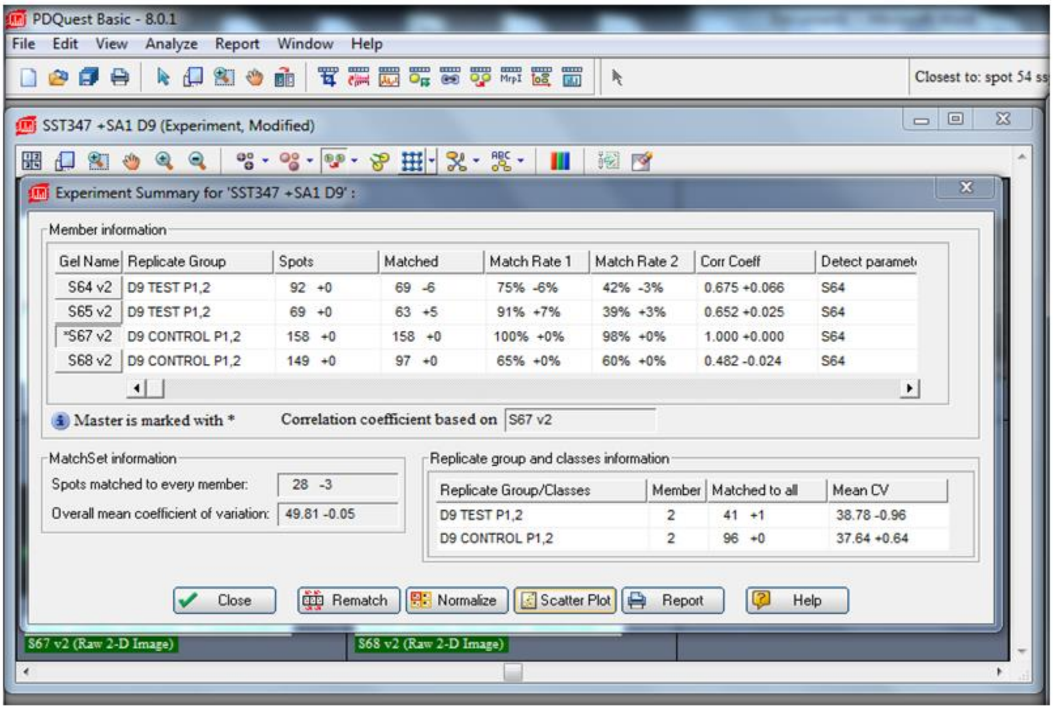
A- Screen capture of inputted parameters for faint and large spots cluster.

B- Screen capture of the results of the image analysis obtained when two groups of gels were compared (SST 347 DAI=9 infested vs uninfested) with the master gel at the far left and the green crosses representing the matched spots.

2.3.7 PDQuest Results

2.3.7.1 Experiment summary

Figure 2.11 shows an example of screen shot of the experiment window displayed after running the software. This is an interactive chart that shows the number of matched protein spots on the gels within the entire experiment based on the user settings. It also allows for changes to be made on some experimental settings such as matching and normalization. The spots column gave the number of spots detected per gel whilst the matched gave the matching total. Match Rate 1 allowed the review of how spots and matching parameters were selected. This displayed the percentage of matched spots relative to the total number, whereas Match Rate 2 showed spots relative to those on the master gel. The summary in Figure 2.11 showed that 92 spots were detected in the second gel for infested SST 347 at day 9 (DAI=9) with 42% of the spots matching those on the master gel. One hundred and forty-nine spots were detected on control gel 2 (S68) with 60% of the spots rmatching those on the master gel. A total of 31 spots were found to match every member.



Member information

Gel Name	Replicate Group	Spots	Matched	Match Rate 1	Match Rate 2	Corr Coeff	Detect paramet
S64 v2	D9 TEST P1,2	92 +0	69 -6	75% -6%	42% -3%	0.675 +0.066	S64
S65 v2	D9 TEST P1,2	69 +0	63 +5	91% +7%	39% +3%	0.652 +0.025	S64
*S67 v2	D9 CONTROL P1,2	158 +0	158 +0	100% +0%	98% +0%	1.000 +0.000	S64
S68 v2	D9 CONTROL P1,2	149 +0	97 +0	65% +0%	60% +0%	0.482 -0.024	S64

Master is marked with *

Correlation coefficient based on: S67 v2

MatchSet information

Spots matched to every member: 28 -3

Overall mean coefficient of variation: 49.81 -0.05

Replicate group and classes information

Replicate Group/Classes	Member	Matched to all	Mean CV
D9 TEST P1,2	2	41 +1	38.78 -0.96
D9 CONTROL P1,2	2	96 +0	37.64 +0.64

Figure 2.11: Results obtained from duplicate gel matching.

The input parameters for spot selection (faint and largest spots) in the PDQuest^{TM} software help to eliminate background particles that could resemble spots as well as the removal of streaks which results in the enhancement of actual protein spots detection. Other features of the software were normalisation of each gel, background noise filtering and speckles removal. To allow spot-to-spot comparisons across gels, a match set was created that included all gel images within an experiment. This match set was the reference gel image (master gel). The software was allowed to create a master gel which was a virtual copy of all the gels in the experiment set and contained all the spots detected from all the gels in that specific experiment (Figure 2.10). As a result, all the identified spots on all gels were included in the master gel and each gel in the experiment was then compared to the relevant master gel. However, a protein spot of interest that could not match to the master gel could be included.

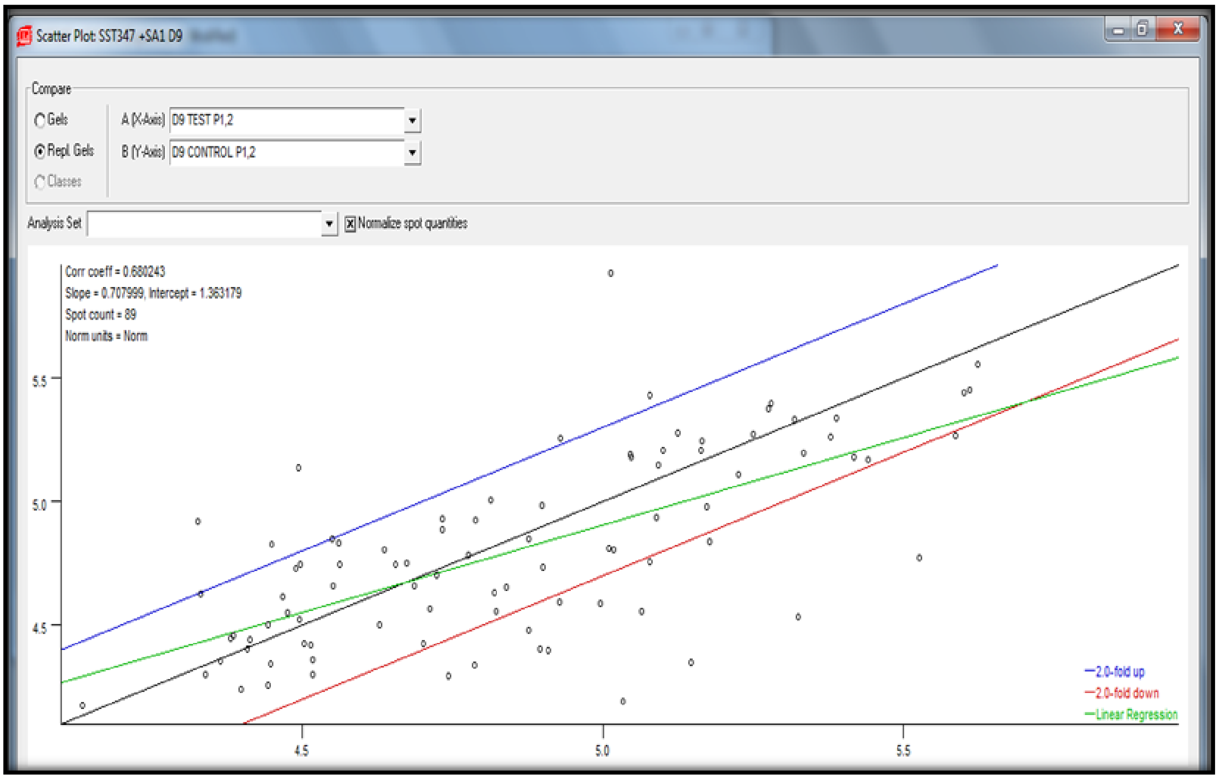
In the experiment summary table (Figure 2.11), the spots column showed the number of spots detected in each gel while the match column indicated how many matched spots were found on each gel. The match Rate 1 showed the percentages of matching to the total number of spots on the gel image. This allowed the review of how the spots and matching parameters were selected. The match rate 2 showed the percentage of matching spots relative to spots included in the master gel. Spots from all gels were added to the master gel that also showed how well the gels contributed. Lastly the correlation coefficient showed that individual gel images matched within the replicate

The scatter plots (Figure 2.12) were also used to represent information obtained from the analysis. The Blue and the red lines showed the border for the 2-fold differences between the protein spot quantities while the green line was an indication of the linear regression that represents correlation between the two groups. The correlation showed how similar the two replicate groups were to each other. For all gels, spot quantities (total pixel intensity within spot boundaries, calculated by image analysis software for Gaussian spots) were normalized to remove variations in spot intensity caused by non-treatment effects. This was represented on a histogram with the spot quantity, based on intensity displayed as a bar. Normalized spot quantity was equal to raw spot quantity expressed as a percentage of the total pixel quantity of all spots in a gel (Zheng *et al*., 2007).

The treated gels were compared with their respective controls to see where major changes occurred. It was necessary to determine the nature of proteins that were up or down regulated during normal wheat growth and development in order to determine whether or not the upregulated or down regulated proteins in stressed plants could be attributed to the stress condition or proteins expressed during normal growth. The results obtained showed that significant changes occurred at 3 hrs, Day 3 and Day 7 post infestation. Based on this, 3 hrs was chosen to look for early responses while Day 3 and Day 7 for late responses. Changes in the wheat leaf proteome in both resistant wheat cultivar (SST 347) and the moderately resistant wheat variety (SST 356) in response to aphid infestation were investigated. The zero time frame (before infestation) served as a control to compare the proteome of individual treatments.

2.3.7.2 Scattered plots

Reproducibility of spots in the duplicate gels was determined from scatter plots generated by PDQuest, an example is shown in Figure 2.12.



Scatter Plot: SST347 +SA1 D9

Compare
○ Gels
● Repl. Gels
○ Classes

A (X-Axis) D9 TEST P1,2
B (Y-Axis) D9 CONTROL P1,2

Analysis Set
☒ Normalize spot quantities

Corr coeff = 0.680243
Slope = 0.707999, Intercept = 1.363179
Spot count = 89
Norm units = Norm

Figure 2.12: Linear regression of reproducibility of 2-DE gels in duplicate after PDQuest analysis. The blue and red lines shows the border for the two fold differences between protein spot quantities while the green line shows a linear regression that represents correlation between the two groups.

2.3.7.3 Quantity graph report

A histogram graph contains the most comprehensive information about a spot and is a useful tool in detecting unmatched spots or differentially expressed spots. Spot quantity based on intensity was displayed as a bar with the bars representing the gels from left to right in the order loaded into the experiment (Figure 2.13). The number in the upper right hand corner is the quantity of the highest bar on the graph; all other bars are proportional to that number. The identified spots were given unique SSP numbers (Standard Spot number) and shown beneath the histograph that provides spot location information. Figure 2.13 below is an example of a quantity graph report with the red bars representing the protein quantities for the infested SST 347 whereas green bars are for uninfested. The presence of only red bar (s) represent proteins that are expressed only in the infested plants and the presence of only green bar (s) represents protein expressed only in the control plants. Differentially expressed proteins are indicated with longer red or green bars. No changes in protein quantity are represented by bars which are approximately the same height. The summary of protein spots detected on gels of SST 347 in duplicates (plants in pot 1 and pot 2) with and without RWA SA1 infestation is as shown in figure 2.14.

○ All spots
● Analysis set: SST 356 +SA1 3HRS (82 spots)
Sort by: ● SSP ○ DSN ○ Trend ○ Quantity
Page number: 1 of 2

Figure 2.13: Screen print of Quantity Graph Report obtained after analysis of the match-set: SST 347 infested vs. uninfested at DAI=9 (only page 1 of 4 shown).

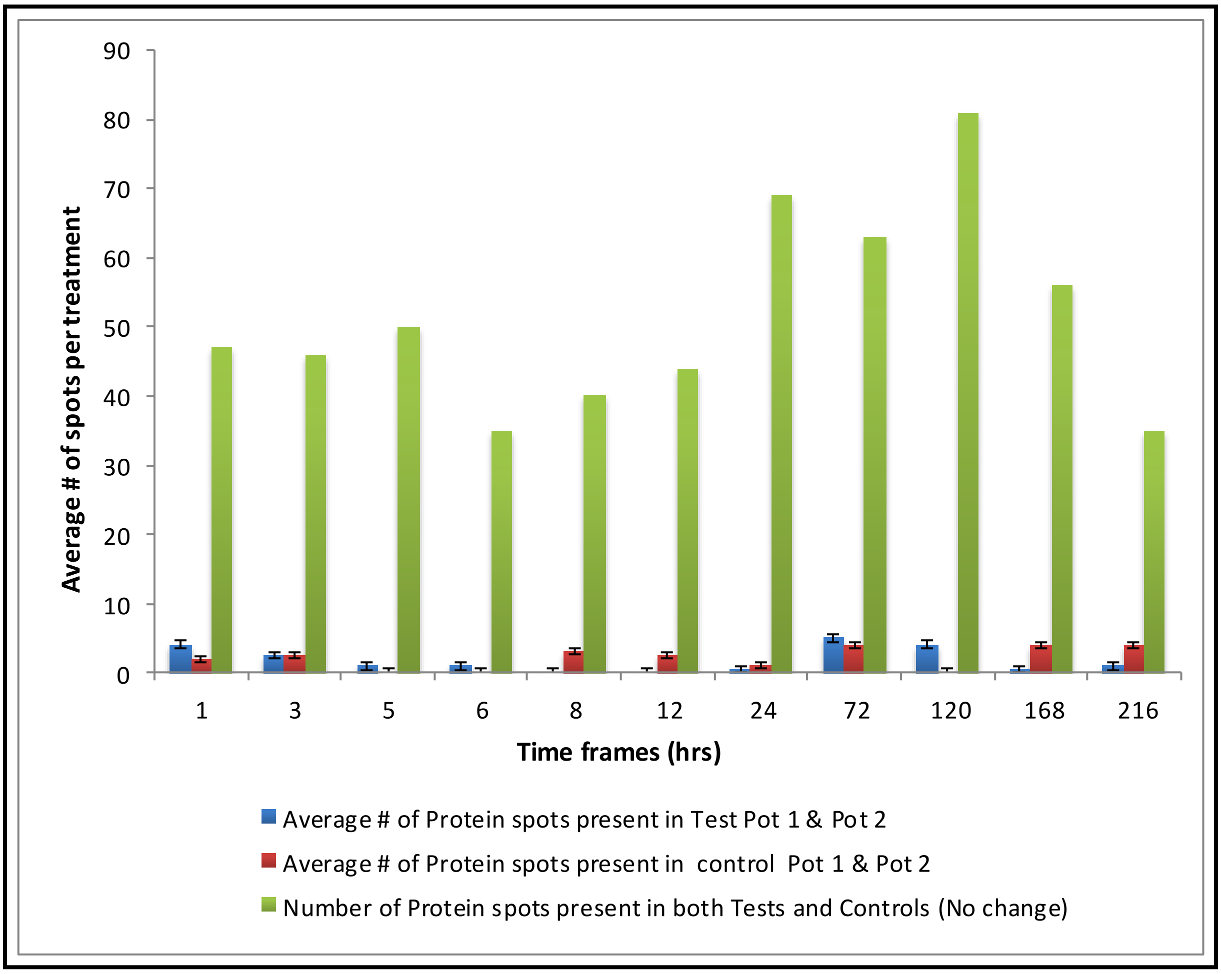


Figure 2.14: Summary of protein spots detected on gels of SST 347 with and without RWA SA1.

2.4 Aphid population growth

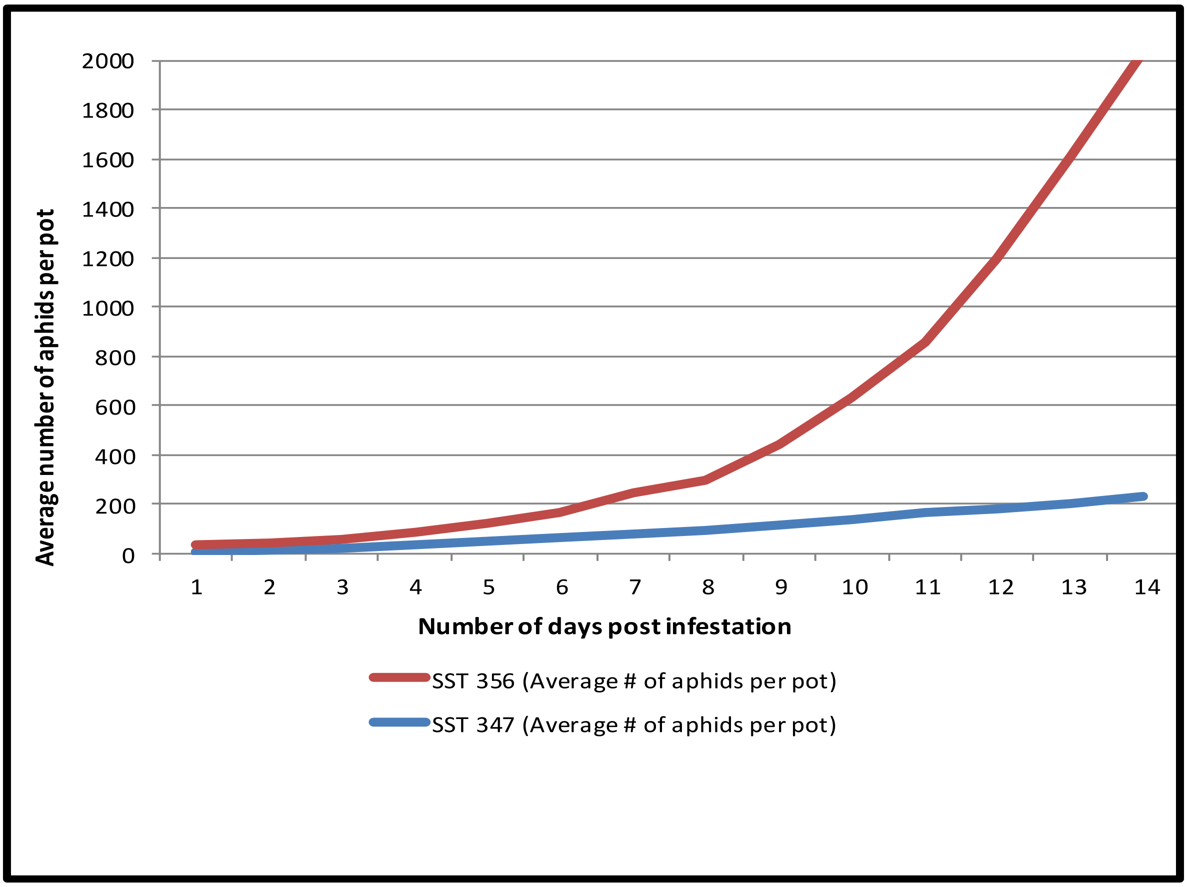


Figure 2.15: RWA SA1 population growth on the moderately resistant (SST 356) and the resistant (SST 347) wheat cultivar.

When RWA SA1 were first introduced onto both the resistant and the moderately resistant wheat cultivars, it took approximately three days for aphids to start reproducing. This period is known as the lag phase, the time in which the aphids settle on their new host and adapt to the new environment. However, a significantly more ($p<0.05$) aphids settled on the moderately resistant cultivar than the resistant cultivar. After adaptation (day 4 &5), the differences in the mean number of aphids was not significant. From day 6 to day 14, RWA SA1 bred faster on the moderately resistant cultivar (SST 356) compared to the resistant cultivar (SST 347) (Figure 2.15) and the differences were statistically significant ($p<0.05$). It has been reported that primary and secondary metabolites found in plant phloem exudates could influence aphid development and reproduction (Awmack and Leatther, 2002; Khan and Port, 2008). Therefore, the resistant cultivar could possess some secondary metabolites that retard the growth of the aphids (although the products of *Dn* gene have not been determined). This is in line with that of Niemeyer (1988) who reported that resistant plant Krwa9 contained higher levels of antibiotic secondary metabolites such as hydroxamic that reduced insect attack while the Kwale line without the RWA resistance was more suitable for aphid development.

2.5 Implication of aphid feeding on plant growth and productivity

Aphids host plants are affected mainly in three ways. Firstly, aphid’s damage the plant by feeding directly on phloem sap and denying the plants essential food materials needed for growth (Dixon, 1988; Miles, 1999). Secondly, they could act as vectors of viruses, spreading associated diseases in crop fields through their mode of feeding (Vickerman and Wratten, 1979; Miller, 1990). Lastly, aphids excrete honeydews on parts of plant where they feed, being sugary, it promotes the growth of saprophytic and pathogenic fungi and bacteria therefore indirectly predisposing the affected part of the plant to infection (Manitoba Agriculture, Food and Rural Initiatives, 2010). It was noticed that with the initial few days of aphid introduction to the plants, no visible damage was detected. However, as the aphids got adapted to their new environment and the population began to build up, damaged to the plant was noticed and the moderately resistant cultivar was almost dead by day 9. This is in line with findings of Hewitt *et al*. (1984) who reported that Russian wheat aphid damaging of wheat usually occur by direct feeding and was noticed when colonies of ten or more aphids invade the plant from the seedling through the head filling stage. As the aphid population continue to build gradually it causes huge damage to the crop field.

It was observed that resistant plants sustained fewer aphids but did exhibit some chlorosis much later. It has been reported that effective wheat host responses against *D. noxia* occur via different modes of resistance including antibiosis, antixenosis, tolerance or a combination of these (Painter, 1958). These could be mediated by specific genetic pathways including signal transduction, ethylene-mediated responses and systemic resistance known to counteract the aphid attack (Botha *et al*., 2010; Smith *et al*., 2010, Marimuthu and Smith, 2012; Liu *et al*., 2011). For the host plant to sustain growth and proliferate under aphid attack, it must be able to recognise the invasion and initiate a defence response (Botha *et al*., 2006; 2010). Failure to do so is associated with elevated stress and early onset of senescence and under severe infestations, even death as observed in the aphid susceptible cultivar.

2.6 Differentially expressed proteins at 3 hrs following RWA SA1 infestation

Comparing the 3 hrs test gels of SST 347 to their controls, there was no difference in the spots identified from 2-DE by PDQuest software. However, comparing the 3 hrs test gels of SST 347 to that of SST 356, four protein spots (A1, A2, A3 & A4) appeared in the former (Figure 2.16A). These proteins could be related to stress and were therefore excised and further analysed by LCMS. From these four spots that were differentially expressed, a total of 26 proteins were identified by mass spectrometry (Figure 2.16B). The Oxygen-evolving enhancer protein1 appeared to be the most abundant protein detected in all four spots; it had the highest percentage coverage of 60.90, 64.92, 56.62 and 59.08 for spot A1, A2, A3 and A4 respectively. The other identified proteins were assumed to have co-migrated with the oxygen-evolving protein 1 (Table 2.3).

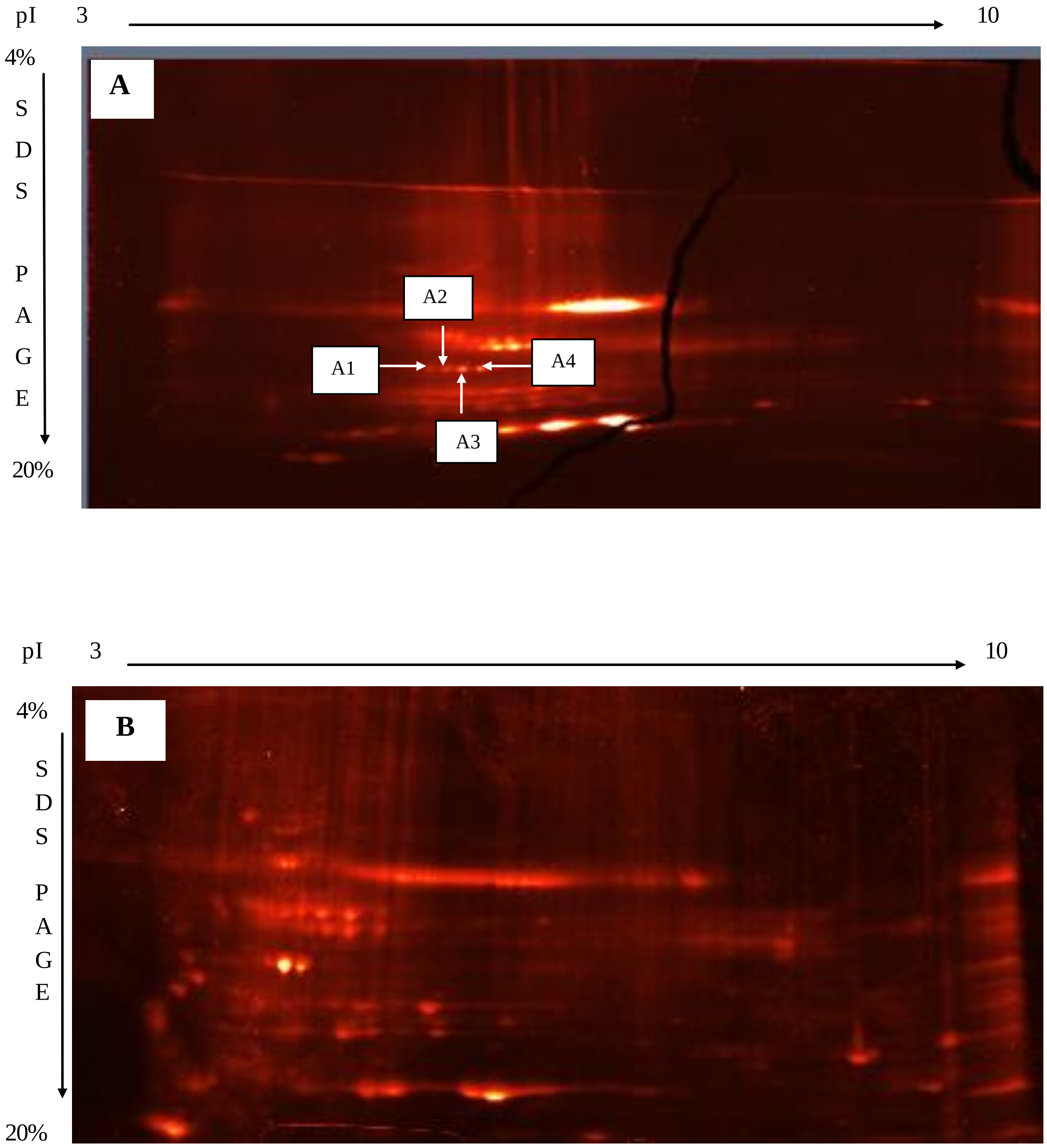


Figure 2.16: 2-D gel of SST 347 (A) at 3 hrs post infestation with 10 RWA SA1. A1-A4; up-regulated protein spots in SST 347 when compared with SST 356 (B) that were excised.

Table 2.3: Proteins identified from the wheat leaf proteome (SST 347) 3 hrs post aphid infestation.

N	Putative protein ID	Accession number	% coverage (95%)	pI/Molecular weight (Dalton)[1]	Function[2]	References
1	Cytochrome f	P05151	3,438000008[b]	8.83/35362.89	Component of the cytochrome b6-f complex, which mediates electron transfer between photosystem II (PSII) and photosystem I (PSI), cyclic electron flow around PSI, and state transitions.	Willey *et al*., 1984 Ogihara *et al*., 2000
2	Histone H2A.2.1	P02276	5,959999934*	10.63/16013.00	Core component of nucleosome. Nucleosomes wrap and compact DNA into chromatin, limiting DNA accessibility to the cellular machineries which require DNA as a template. Histones thereby play a central role in transcription regulation, DNA repair, DNA replication and chromosomal stability. DNA accessibility is regulated via a complex set of post-translational modifications of histones, also called histone code, and nucleosome remodeling.	Rodrigues *et al*., 1979; 1988 Tabat *et al*., 1984 Modro *et al*., 1988 Huh *et al*., 1997 Green *et al*., 1990 Yang *et al*., 1995
3	Protein H2A.7	Q43312	6,716000289*	10.23/13932.23		
4	Protein H2A.6	Q43214	6,08099997*	10.56/ 15633.41		
5	Protein H2A.5	Q43213	6,207000092*	10.62/15388.09		
6	Histone H2A.4	Q43208	6,667000055*	10.05/ 14102.25		
7	Histone H3.2	P68428	5,14700003[c]	11.29/15283.90		
8	Histone H2B2	P05621	5,999999866[a]	10.19/ 16226.50		
9	Histone H2B.1	P27807	5,999999866[b]	10.25/16236.62		
10	Protein H2B.4	Q43215	6,667000055[a]	10.08/ 14816.46		
11	Protein H2B.5	Q43216	6,617999822[a]	10.03/ 14968.62		
12	Histone 32	P68428	5,14700003[c]	11.29/ 15283.90		
13	Histone H2B.3	Q43217	6,521999836[a]	10.0/15104.73		
14	Oxygen-evolving	Q00434	13,17999959*	27269.68	May be involved in the regulation of	James and

	enhancer protein 2, chloroplastic		16,67000055[a] 18,60000044[b] 7,751999795[c]		photosystem II.	Robinson, 1991
15	Thioredoxin M-type, chloroplastic	Q9ZP21	5,143000185* 10,28999984[c]	8.67/19132.37	Participates in various redox reactions through the reversible oxidation of the active center dithiol to a disulfide. The M form is known to activate NADP-malate dehydrogenase (By similarity).	Dyer *et al.*, 1998
16	Photosystem II D1protein	P12463	19,8300004* 22,10000008[a,b] 12,46000007[c]	5.21/ 38920.62	Photosystem II (PSII) is a light-driven water: plastoquinone oxidoreductase that uses light energy to abstract electrons from H(2)O, generating O(2) and a proton gradient subsequently used for ATP formation. It consists of a core antenna complex that captures photons, and an electron transfer chain that converts photonic excitation into a charge separation. The D1/D2 (PsbA/PsbA) reaction center heterodimer binds P680, the primary electron donor of PSII as well as several subsequent electron acceptors. D2 is needed for assembly of a stable PSII complex.	Hanley *et al.*, 1988 Ogihara *et al.*, 2000
17	Photosystem II D2 protein	Q36814	14,15999979*,[a] 14,15999979[b] 9,064999968[c]	39470.38		

18	ATP synthase subunit alpha, mitochondrial	P12862	1,964999922[c]	5.70/55264.20	Mitochondrial membrane ATP synthase (F_1F_0 ATP synthase or Complex V) produces ATP from ADP in the presence of a proton gradient across the membrane which is generated by electron transport complexes of the respiratory chain. F-type ATPases consist of two structural domains, F_1 - containing the extramembraneous catalytic core, and F_0 - containing the membrane proton channel, linked together by a central stalk and a peripheral stalk. During catalysis, ATP synthesis in the catalytic domain of F_1 is coupled via a rotary mechanism of the central stalk subunits to proton translocation. Subunits alpha and beta form the catalytic core in F_1. Rotation of the central stalk against the surrounding alpha$_3$beta$_3$ subunits leads to hydrolysis of ATP in three separate catalytic sites on the beta subunits. Subunit alpha does	Schulte *et al*., 1989

					not bear the catalytic high-affinity ATP-binding sites (By similarity).	
19	ATP synthase subunit beta, chloroplastic	P20858	31,92999959* 21,0800007^{a} 7,631000131^{b} 15,86000025^{c}	5.06/53857.48	Produces ATP from ADP in the presence of a proton gradient across the membrane. The catalytic sites are hosted primarily by the beta subunits.	Howe *et al.*, 1985 Ogihara *et al.*, 2000
20	Sedoheptulose-1,7-bisphosphatase, chloroplastic	P46285	28,7499994* 22,65000045^{a} 12,47000024^{b}	6.04/42060.82	Catalytic activity: Sedoheptulose 1, 7-bisphosphate + H_2O = sedoheptulose 7-phosphate + phosphate.	Raines *et al.*, 1992 Miles *et al.*, 1993
21	Ribulose bisphosphate carboxylase large chain	P11383	11,11000031*^{,a} 8,805000037^{b} 9,853000194^{c}	6.22/52851.13	RuBisCO catalyzes two reactions: the carboxylation of D-ribulose 1, 5-bisphosphate, the primary event in carbon dioxide fixation, as well as the oxidative fragmentation of the pentose substrate in the photorespiration process. Both reactions occur simultaneously and in competition at the same active site.	Broglie *et al.*, 1983 Smith *et al.*, 1983 Terachi *et al.*, 1987 Houtz *et al.*, 1989 Mehta *et al.*,
22	Ribulose bisphosphate carboxylase small chain PW9, chloroplastic	P26667	23,43000025*^{,b} 18,86000037^{a} 38,85999918^{c}	8.52/19454.45		

23	Ribulose bisphosphate carboxylase small chain PWS4.3 , chloroplastic	P00871	13,22000027*^{,b} 27,00999975^{a} 28,74000072^{c}	8.99/19417.36		1992 Ogihara *et al*., 1991; 2000 Niu, 2003
24	Ribulose bisphosphate carboxylase small chain clone 512 (Fragment)	P07398	20,35000026*^{,a,,b} 44,24999952^{c}	5.84/ 13055.05		
25	Oxygen-evolving enhancer protein 1, chloroplastic	P27665	60,92000008* 64,92000222^{a} 56,62000179^{b} 59,07999873^{c}	8.73/34740.37	Stabilizes the manganese cluster which is the primary site of water splitting.	Meadows *et al*., 1991
26	RuBisCO large subunit-binding protein subunit alpha, chloroplastic (Fragment)	P08823	10,67999974* 15,64999968^{a}	4.83/57520.59	This protein binds RuBisCO small and large subunits and is implicated in the assembly of the enzyme oligomer.	Hemmingsen *et al*., 1988

* % coverage of proteins spot A1, ^{a}% coverage of proteins spot A2, ^{b}% coverage of proteins spot A3, ^{C}% coverage of proteins spot A4. 1&2 (p^{I}/Molecular weight and function of the proteins) were obtained from www.ExPASy.org.

2.7 Differentially expressed proteins at day 3 following RWA SA1 infestation

When gels of SST 347 at Day 3 following aphid feeding were compared to their controls, there was no difference in the protein spots identified. On the other hand, a comparison of gels of SST 347 to those of SST 356, 9 proteins were differentially regulated and could be attributed to stress imposed by aphid feeding (Figure 2.17A). A total of 28 proteins were identified from the 9 spots (B1, B2, B3, B4, B5, B6, B7, B8 and B9). Proteins most likely to represent the spots include Oxygen evolving enhancer protein 2 (B1, B2, B4, B5 and B6), Oxygen-evolving enhancer protein1 (B7), Ribulose biphosphate carboxylase slarge chain (B3) and Phosphoglycerate kinase (B8 and B9). The other proteins must have co-migrated with these (Table 2.4).

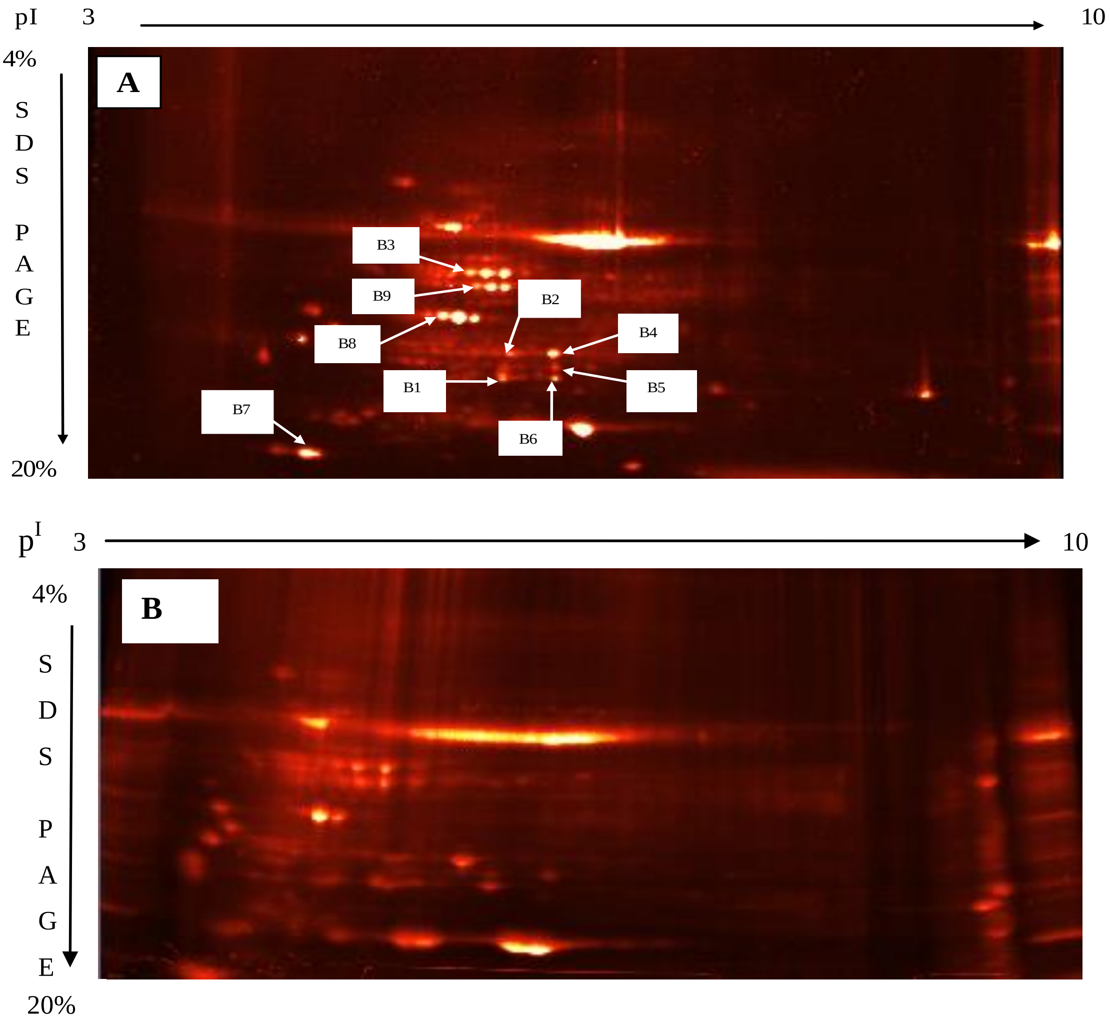


Figure 2.17: 2-D gel of SST 347 (A) at Day 3 post infestation with 10 RWA SA1 showing regulated protein spots when compared with SST 356 (B) that were excised.

Table 2.4: Proteins identified from the wheat leaf proteome (SST 347) Day 3 post aphid infestation

N	Putative Protein ID	Accession number	% coverage (95%)	pI/Molecular weight (Dalton)[1]	Function[2]	References
1	Oxygen-evolving enhancer protein 2, chloroplastic	Q00434	65.11999965* 29.06999886[a] 40.31000137[b] 64.34000134[c] 43.41000021[d] 7.751999795[e] 17.04999954[g] 8.139999956[h]	8.84/27269.68	Maybe involved in the regulation of photosystem II	James and Robinson, 1991
2	Cytochrome b6-f complex iron-sulfur subunit, chloroplastic	Q7X9A6	5.404999852[b] 13.51000071[c]	8.47/23726.26	Component of the cytochrome b6-f complex, which mediates electron transfer between photosystem II (PSII) and photosystem I (PSI), cyclic electron flow around PSI, and state transitions.	Lu *et al*., 2002
3	Histone H2B.3	Q43217	6.521999836[b,c]	10.08/15104.73	Core component of nucleosome. Nucleosomes wrap and compact DNA into chromatin, limiting DNA accessibility to the cellular machineries which require	Brandt *et al*., 1988 Green *et al*., 1990 Yang *et al*., 1995
4	Histone H2B.2	P05621	5.999999866[*,c]	10.19/16226.50		

5	Histone H2B.4	Q43215	6.667000055[b,c]	10.08/14816.46	DNA as a template. Histones thereby play a central role in transcription regulation, DNA repair, DNA replication and chromosomal stability. DNA accessibility is regulated via a complex set of post-translational modifications of histones, also called histone code, and nucleosome remodeling.	
6	Histone H2B.5	Q43216	6.617999822[b,c]	10.03/14968.62		
7	Histone H2B.1	P27807	5.92099987[*,c]	10.00/16433.26		
8	Serpin-Z1A	Q41593	2.512999997[d]	5.60/43118.31	Inhibits chymotrypsin and cathepsin G in vitro.	Rosenkrands *et al*., 1994 Dahl *et al*., 1996 Rasmussen *et al*., 1996 Oestergaard *et al*., 1996; 2000
9	Serpin-Z1B	P93693	2.50599999[d]	5.44 /43032.95		
10	Serpin-Z1C	Q9ST58	2.512999997[d]	5.62/42881.85		
11	Phosphoglycerate kinase, chloroplastic	P12782	2.707999945[f] 32.91999996[g] 65.63000083[h]	6.57/49839.53	Catalytic activity: ATP + 3-phospho-D-glycerate = ADP + 3-phospho-D-glyceroyl phosphate.	Longstaff *et al*., 1989 Jones *et al*., 1995
12	S-adenosylmethionine synthase	B0LXM0	3.787999973[h]	5.55/43179.90	Catalyzes the formation of S-adenosylmethionine from methionine and ATP. The overall synthetic reaction is composed of two sequential steps, AdoMet	Wang *et al*., 2008

					formation and the subsequent tripolyphosphate hydrolysis which occurs prior to release of AdoMet from the enzyme (By similarity).	
13	2-Cys peroxiredoxin BAS1, chloroplastic (Fragment)	P80602	6.667000055[d,f,h]	5.71/23326.68	May be an antioxidant enzyme particularly in the developing shoot and photosynthesizing leaf.	Tsunoyama & Toyoshima, 1997 Tsunoyama *et al*., 1996
14	Photosystem II D1protein	P12463	9.347999841[f] 8.782000095[h]	5.21/38920.62	Photosystem II (PSII) is a light-driven water: plastoquinone oxidoreductase that uses light energy to abstract electrons from H(2)O, generating O(2) and a proton gradient subsequently used for ATP formation. It consists of a core antenna complex that captures photons, and an electron transfer chain that converts photonic excitation into a charge separation. The D1/D2 (PsbA/PsbA) reaction center heterodimer binds P680, the primary electron donor of PSII as well as several subsequent electron acceptors.	Hanley *et al*., 1988 Ogihara *et al*., 2000
15	Photosystem II D2 protein	Q36814	9.064999968[f]	5.34/39470.38		

					D2 is needed for assembly of a stable PSII complex.	
16	ATP synthase subunit alpha, mitochondrial	P12862	2.35799998[a] 6.482999772[c] 11.98000014[d] 8.643999696[g]	5.70/55264.20	Mitochondrial membrane ATP synthase (F_1F_0 ATP synthase or Complex V) produces ATP from ADP in the presence of a proton gradient across the membrane which is generated by electron transport complexes of the respiratory chain. F-type ATPases consist of two structural domains, F_1 - containing the extramembraneous catalytic core, and F_0 - containing the membrane proton channel, linked together by a central stalk and a peripheral stalk. During catalysis, ATP synthesis in the catalytic domain of F_1 is coupled via a rotary mechanism of the central stalk subunits to proton translocation. Subunits alpha and beta form the catalytic core in F_1. Rotation of the central stalk against the surrounding $alpha_3beta_3$ subunits leads to hydrolysis of ATP in three separate	Schutle *et al.*, 1989

					catalytic sites on the beta subunits. Subunit alpha does not bear the catalytic high-affinity ATP-binding sites (By similarity).	
17	ATP synthase subunit beta, chloroplastic	P20858	6.42599985* 9.036000073[c] 25.90000033[d] 26.51000023[f] 26.10000074[g] 41.37000144[h]	5.06/53857.04	Produces ATP from ADP in the presence of a proton gradient across the membrane. The catalytic sites are hosted primarily by the beta subunits.	Howe *et al*., 1985 Ogihara *et al*., 2000
18	ATP synthase subunit alpha, chloroplastic	P12112	20.63000053* 22.22000062[a] 3.968000039[b] 12.70000041[c] 16.47000015[d] 11.11000031[h]	6.11/55295.52		
19	ADP,ATP carrier protein 2, mitochondrial	Q41630	6.34400025[g]	9.78/35790.30	Tramnsmembrane transport. Catalyses the exchange of ADP and ATP across the mitochondrial inner membrane	Iacobazzi *et al*., 1996

20	ADP,ATP carrier protein 1, mitochondrial	Q41629	6.34400025^{g}	9.80/35961.63		
21	Ribulose bisphosphate carboxylase small chain clone 512 (Fragment)	P07398	$20.35000026^{*,b,c,d,f,g,h}$ 6.194999814^{a} 14.15999979^{b}	5.84/13055.05	RuBisCO catalyzes two reactions: the carboxylation of D-ribulose 1, 5-bisphosphate, the primary event in carbon dioxide fixation, as well as the oxidative fragmentation of the pentose substrate. Both reactions occur simultaneously and in competition at the same active site (By similarity).	Broglie *et al*., 1983 Smith *et al*., 1983 Terachi *et al*., 1987 Houtz *et al*., 1989 Mehta *et al*., 1992 Ogihara *et al*., 1991; 2000 Niu, 2003
22	Ribulose bisphosphate carboxylase small chain PW9, chloroplastic	P26667	$18.86000037^{*,c,d,g}$ 3.999999911^{a} $23.43000025^{f,h}$	8.52/19454.45		
23	Ribulose bisphosphate carboxylase large chain	P11383	16.34999961^{*} 12.1600002^{a} 14.85999972^{b} 24.74000007^{c} 15.51000029^{d} 8.805000037^{f} 1.88699998^{g} 9.223999828^{h}	6.22/52851.13		
24	Ribulose bisphosphate carboxylase small	P00871	$13.22000027^{*,c,d,f,g,h}$	8.99/19417.36		

	chain PWS4.3, chloroplastic		8.620999753[a] 4.597999901[b]			
25	Oxygen-evolving enhancer protein 1, chloroplastic	P27665	50.15000105[f]	8.73/34740.37	Stabilizes the manganese cluster which is the primary site of water splitting.	Meadows *et al.*, 1991
26	Phosphoribulokinase, chloroplastic	P26302	2.969999984[g] 37.38000095[h]	5.72/45141.39	Catalytic activity: ATP + D-ribulose 5-phosphate = ADP + D-ribulose 1, 5-bisphosphate.	Raines *et al*., 1989 Lloyd *et al.*, 1991
27	Sedoheptulose-1,7-bisphosphatase, chloroplastic	P46285	16.03000015[f] 29.01000082[g] 18.07000041[h]	6.04/42060.82	Catalytic activity: Sedoheptulose 1, 7-bisphosphate + H_2O = sedoheptulose 7-phosphate + phosphate.	Raines *et al*., 1992 Miles *et al.*, 1993
28	RuBisCO large subunit-binding protein subunit alpha, chloroplastic (fragment)	P08823	8.839999884[f]	4.83/57520.59	This protein binds RuBisCO small and large subunits and is implicated in the assembly of the enzyme oligomer.	Hemmingsen *et al*., 1988

***** % coverage of proteins spot B1, **[a]**% coverage of proteins spot B2, **[b]**% coverage of proteins spot B3, **[C]**% coverage of proteins spot B4, [d]% coverage of proteins spot B5, [e]% coverage of proteins spot B6, [f]% coverage of proteins spot B7, [g]% coverage of proteins spot B8, [h]% coverage of proteins spot B9. 1&2 (pI/Molecular weight and function of the proteins) were obtained from www.ExPASy.org.

2.8 Differentially Expressed proteins at Day 7 following RWA SA1 infestation

There was no difference in proteins expressed between the test and the respective controls of SST 356 and SST 347. However, 5 protein spots were present on the test gels of SST 356 and absent in SST 347 test. On the other hand, 7 protein spots were present in SST 347 and absent in SST 356. From the 5 spots (C1, C2, C3, C4, C5) (Figure 2.18 B), 45 proteins (Table 2.5) were identified while 22 proteins were identified from the 7 spots (D1, D2, D3, D4, D5, D6, D7) (Figure 2.18 A, Table 2.6). The most likely proteins representing the spots include Ribulose bisphosphate carboxylase small chain clone 512 (C1), Oxygen-evolving enhancer protein 2 (C2, C3 and C4), ATP synthase subunit beta (C5, D5, D6, D7), Oxygen-evolving enhancer protein1 (D1), phosphoglycerate kinase (D2, D3 and D4) (Table 2.5 and Table 2.6).

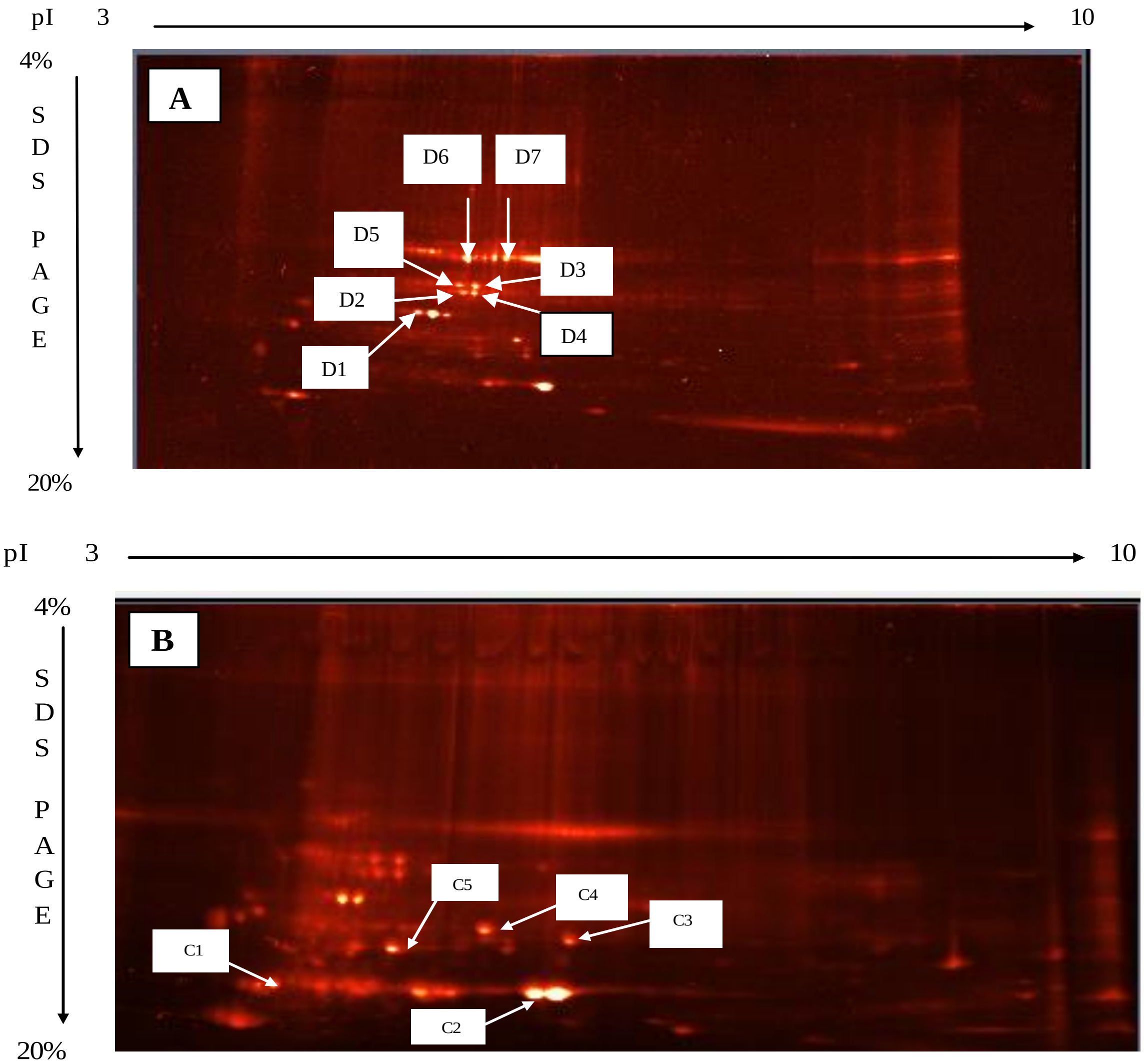


Figure 2.18: Excised protein spots from 2-D gel at Day 7 post infestation with 10 RWA SA1. D1-D7 were up-regulated protein spots in SST 347(A) while C1-C4 were up regulated protein spots in SST 356 (B).

Table 2.5: Proteins identified from the wheat leaf proteome (SST 356) Day 7 post aphid infestation

N	Putative protein ID	Accession number	% coverage (95%)	pI/Molecular weight (Dalton)[1]	Function[2]	References
1	Oxalate oxidase GF-3.8	P26759	8,482000232[a]	8.57/23562.24	Produces developmental and stress-related release of hydrogen peroxide in the apoplast. May play an important role in several aspects of plant growth and defense mechanisms.	Lane *et al*., 1991; 1993
2	Profilin-3	P49234	24,42999929[e]	4.88/14141.17	Binds to actin and affects the structure of the cytoskeleton. At high concentrations, profilin prevents the polymerization of actin, whereas it enhances it at low concentrations. By binding to PIP2, it inhibits the formation of IP3 and DG.	Rihs *et al*., 1994
3	Profilin-1	P49232	24,42999929[e]	4.91/14224.30		
4	Profilin-2	P49233	24,42999929[e]	4.92/142234.21		
5	Cytochrome b559 subunit alpha	P69386	12.04999983*[a,b]	4.64/9444.60	This b-type cytochrome is tightly associated with the reaction center of photosystem II (PSII). PSII is light-driven water: plastoquinone oxidoreductase that uses light energy to abstract electrons from H_2O, generating O_2 and a proton gradient subsequently used for ATP	Hird *et al*., 1986 Ikeuchi *et al*., 1989 Webber *et al*., 1989 Ogihara *et al*., 2000

					formation. It consists of a core antenna complex that captures photons, and an electron transfer chain that converts photonic excitation into a charge separation	
6	Cytochrome b6	P60162	9,766999632[a] 25,11999905[b]	8.89/24182.58	Component of the cytochrome b6-f complex, which mediates electron transfer between photosystem II (PSII) and photosystem I (PSI), cyclic electron flow around PSI, and state transitions.	Hird *et al*., 1991 Lu *et al*., 2002
7	Cytochrome b6-f complex iron-sulfur subunit, chloroplastic	Q7X9A6	6.306000054* 46,84999883[b]	8.47/23726.26		
8	Oxygen-evolving enhancer protein 2, chloroplastic	Q00434	27.1299988* 65,11999965[a] 64,34000134[b]	8.84/27269.68	Maybe involved in the regulation of photosystem II	James and Robinson, 1991
9	ATP synthase epsilon chain, chloroplastic	P69443	27.00999975*[,e] 8,028999716[c]	5.20/15217.63	Produces ATP from ADP in the presence of a proton gradient across the membrane	Howe *et al*., 1985 Ogihara *et al*., 2000
10	Protein H2A.5	Q43213	6.207000092*[,c,e]	10.62/15388.09	Core component of nucleosome. Nucleosomes wrap and compact DNA into chromatin, limiting DNA accessibility to the cellular machineries which require DNA as a template. Histones thereby play a central role in	James and Robinson, 1991 Rodrigues *et al*., 1979; 1988 Tabata *et al*., 1984 Tabata & Iwabuchi,
11	Histone H2A.1	P02275	6.164000183*[,c,e]	10.66/15586.40		
12	Protein H2A.6	Q43214	6.08099997*[,c,e]	10.56/15633.41		
13	Histone H2A.	P02277	5.959999934*	10.72/16070.10		

14	Histone H2A.2.1	P02276	5.959999934*^{,c,e}	10.6/316013.00	transcription regulation, DNA repair, DNA replication and chromosomal stability. DNA accessibility is regulated via a complex set of post-translational modifications of histones, also called histone code, and nucleosome remodeling.	1984 Modro *et al*., 1988 Brandt *et al*., 1988 Howe *et al*., 1985; 1988 Green *et al*., 1990 Huh *et al*., 1995 Yang *et al*., 1991; 1995 Lu *et al*., 2002
15	Histone H2A.4	Q43208	6.667000055*^{,c,e}	10.05/14102.25		
16	Histone H2B.5	Q43216	6,617999822^{a,e} 17,64999926^{b}	10.03/14968.62		
17	Histone H2B.3	Q43217	6,521999836^{a,e} 17,38999933^{b}	10.08/15104.73		
18	Histone H2B.2	P05621	5,999999866^{a,e} 15,99999964^{b}	10.19/16226.50		
19	Histone H2B.1	P27807	5,92099987^{a,e} 15,79000056^{c}	10.00/16433.26		
20	Protein H2A.7	Q43312	6.716000289*^{,c,e}	10.23/13932.23		
21	Histone H4 variant TH011	P62785	29.12999988* 17,47999936^{d} 7,767000049^{e}	11.48/11409.38		
22	Histone H2A.2.2	P02277	5,959999934^{c,e}	10.72/16070.10		
23	Histone H4 variant TH091	P62786	29.12999988* 17,47999936^{d} 7,767000049^{e}	11.33/11467.42		

24	Histone H2B.4	Q43215	6,667000055[a,c,e]	10.08/14816.46		
25	Histone 3.2	P68428	16,91000015[e]	11.29/15283.90		
26	50S ribosomal protein L16, chloroplastic	Q95H50	16,91000015[e]	11.62/15490.27	Interacting selectively and non-covalently with ribosomal RNA.The cellular metabolic process in which a protein is formed, using the sequence of a mature mRNA molecule to specify the sequence of amino acids in a polypeptide chain. Translation is mediated by the ribosome, and begins with the formation of a ternary complex between aminoacylated initiator methionine tRNA, GTP, and initiation factor 2, which subsequently associates with the small subunit of the ribosome and an mRNA. Translation ends with the release of a polypeptide chain from the ribosome.	Ogihara *et al.*, 2000
27	2-Cys peroxiredoxin BAS1, chloroplastic (Fragment)	P80602	19,51999962[a] 21,43000066[b] 6,667000055[e]	5.71/23326.68	May be an antioxidant enzyme particularly in the developing shoot and photosynthesizing leaf.	Tsunoyama *et al.*, 1996 Tsunoyama & Toyoshima, 1997

28	ATP synthase subunit alpha, mitochondrial	P12862	15,71999937[c] 4,126000032[d,e]	5.70/55264.20	Mitochondrial membrane ATP synthase (F1F0 ATP synthase or Complex V) produces ATP from ADP in the presence of a proton gradient across the membrane which is generated by electron transport complexes of the respiratory chain. F-type ATPases consist of two structural domains, F1 - containing the extramembraneous catalytic core, and F0 - containing the membrane proton channel, linked together by a central stalk and a peripheral stalk. During catalysis, ATP synthesis in the catalytic domain of F1 is coupled via a rotary mechanism of the central stalk subunits to proton translocation. Subunits alpha and beta form the catalytic core in F1. Rotation of the central stalk against the surrounding alpha3beta3 subunits leads to hydrolysis of ATP in three separate catalytic sites on the beta subunits. Subunit alpha does not	Schulte *et al*., 1989

					bear the catalytic high-affinity ATP-binding sites (By similarity).	
29	Phosphoglycerate kinase, chloroplastic	P12782	3.542000055*	6.57/49839.53	Catalytic activity: ATP + 3-phospho-D-glycerate = ADP + 3-phospho-D-glyceroyl phosphate.	Longstaff *et al.*, 1989 Jones *et al.*, 1995
30	Photosystem II CP47 reaction center protein	P24065	2.164999954*	6.06/56092.03	One of the components of the core complex of photosystem II (PSII). It binds chlorophyll and helps catalyze the primary light-induced photochemical processes of PSII. PSII is a light-driven water:plastoquinone oxidoreductase, using light energy to abstract electrons from H_2O, generating O_2 and a proton gradient subsequently used for ATP formation	Gray *et al.*, 1990 Hird *et al.*, 1991 Ogihara *et al.*, 2000
31	Thioredoxin M-type, chloroplastic	Q9ZP21	43,99999976[e]	8.67/19132.37	Participates in various redox reactions through the reversible oxidation of the active center dithiol to a disulfide. The M form is known to activate NADP-malate	Dyer *et al.*, 1998

					dehydrogenase	
32	ATP synthase subunit beta, chloroplastic	P20858	19,28000003[*] 26,71000063[a] 2,410000004[c] 21,0800007[d]	6.11/55295.52	Produces developmental and stress-related release of hydrogen peroxide in the apoplast. May play an important role in several aspects of plant growth and defence mechanisms.	Howe *et al.*, 1985 Ogihara *et al.*, 2000
33	Cysteine synthase	P38076	17,54000038[a] 3,691999987[c]	5.46/34114.38	Catalytic activity: O-acetyl-L-serine + hydrogen sulfide = L-cysteine + acetate.	Youssefian *et al.*, 1993
34	Oxalate oxidase GF-2.8	P15290	4,018000141[a]	6.42/23612.30	Produces developmental and stress-related release of hydrogen peroxide in the apoplast. May play an important role in several aspects of plant growth and defence mechanisms.	Lane *et al.*, 1991 Dratewka-Kos *et al.*, 1983 Berna and Bernier, 1999
35	ATP synthase subunit alpha, chloroplastic	P12112	43,84999871[a] 23,01999927[b] 6,746000051[d]	6.11/55295.52	Produces ATP from ADP in the presence of a proton gradient across the membrane. The alpha chain is a regulatory subunit	Howe *et al.*, 1985 Ogihara *et al.*, 2000
36	Photosystem I iron-sulfur center	P69415	11,11000031[a]	6.51/8899.32	Apoprotein for the two 4Fe-4S centers FA and FB of photosystem I (PSI); essential for photochemical activity. FB is the terminal electron acceptor of PSI,	Dunn and Gray, 1988 Ogihara *et al.*, 2000

					donating electrons to ferredoxin. The C-terminus interacts with PsaA/B/D and helps assemble the protein into the PSI complex. Required for binding of PsaD and PsaE to PSI. PSI is a plastocyanin-ferredoxin oxidoreductase, converting photonic excitation into a charge separation, which transfers an electron from the donor P700 chlorophyll pair to the spectroscopically characterized acceptors A0, A1, FX, FA & FB in turn	
37	Chlorophyll a-b binding protein, chloroplastic	P04784	9,774000198[a]	5.67/28264.35	The light-harvesting complex (LHC) functions as a light receptor, it captures and delivers excitation energy to photosystems with which it is closely associated.	Lamppa *et al*., 1985
38	Ribulose bisphosphate carboxylase large chain	P11383	13.84000033* 31,45000041[a] 42,77000129[b] 4,193000123[d] 35,6400013[e]	6.22/52851.13	RuBisCO catalyzes two reactions: the carboxylation of D-ribulose 1, 5-bisphosphate, the primary event in carbon dioxide fixation, as well as the oxidative fragmentation of the pentose substrate.	Broglie *et al*., 1983 Smith *et al*., 1983 Terachi *et al*., 1987 Houtz *et al*., 1989 Mehta *et al*., 1992

39	Ribulose bisphosphate carboxylase small chain clone 512 (Fragment)	P07398	100* 38,94000053[a,e] 14,15999979[d]	5.84/13055.05	Both reactions occur simultaneously and in competition at the same active site	Ogihara *et al.*, 1991; 2000 Niu, 2003
40	Ribulose bisphosphate carboxylase small chain PWS4.3, chloroplastic	P00871	73.55999947* 25,29000044[a] 13,22000027[c] 9,194999933[d] 25,29000044[e]	8.99/19417.36		
41	Ribulose bisphosphate carboxylase small chain PW9, chloroplastic 1	P26667	73.1400013* 35,42999923[a] 23,43000025[b] 14,85999972[d,e] 35,42999923[e]	8.52/19454.45		
42	Chlorophyll a-b binding protein, chloroplastic	P04784	6,767000258[c]	5.67/28264.35	The light-harvesting complex (LHC) functions as a light receptor, it captures and delivers excitation energy to photosystems with which it is closely associated.	Lamppa *et al.*, 1985
43	Phosphoglycerate kinase, chloroplastic	P12782	1,875000075[a] 3,542000055[d] 19,37000006[e]	6.57/49839.53	Catalytic activity: ATP + 3-phospho-D-glycerate = ADP + 3-phospho-D-glyceroyl phosphate.	Longstaff *et al.*, 1989

						Jones *et al*., 1995
44	RuBisCO large subunit-binding protein subunit alpha, chloroplastic (Fragment)	P08823	2,02600006[e]	4.83/57520.59	This protein binds RuBisCO small and large subunits and is implicated in the assembly of the enzyme oligomer.	Hemmingsen *et al*., 1988
45	Cytochrome b559 subunit alpha	P69386	12,04999983[a]	4.64/9444.60	This b-type cytochrome is tightly associated with the reaction center of photosystem II (PSII). PSII is a light-driven water: plastoquinone oxidoreductase that uses light energy to abstract electrons from H_2O, generating O_2 and a proton gradient subsequently used for ATP formation. It consists of a core antenna complex that captures photons, and an electron transfer chain that converts photonic excitation into a charge separation	Hird *et al*., 1986 Webber *et al*., 1989 Ikeuchi *et al*., 1989 Ogihara *et al*., 2000

* % coverage of proteins spot C1, [a]% coverage of proteins spot C2, [b]% coverage of proteins spot C, [C]% coverage of proteins spot C4, [d]% coverage of proteins spot C5. 1&2 (pI/Molecular weight and function of the proteins) were obtained from www.ExPASy.org.

Table 2.6: Proteins identified from the wheat leaf proteome (SST 347) Day 7 post aphid infestation

N	Protein (s) identified	Accession number	% coverage (95%)	pI/Molecular weight (Dalton)[1]	Function[2]	References
1	Adenosylhomocysteinase	P32112	1.443000045[d]	5.65/53436.52	Adenosylhomocysteine is a competitive inhibitor of S-adenosyl-L-methionine-dependent methyl transferase reactions; therefore adenosylhomocysteinase may play a key role in the control of methylations via regulation of the intracellular concentration of adenosylhomocysteine.	Richards and Gardner, 1993
2	Photosystem II CP47 reaction center protein	P24065	2.164999954[d]	6.06/56092.03	One of the components of the core complex of photosystem II (PSII). It binds chlorophyll and helps catalyze the primary light-induced photochemical processes of PSII. PSII is a light-driven water:plastoquinone oxidoreductase, using light energy to abstract electrons from H_2O, generating O_2 and a proton gradient subsequently used for ATP formation	Gray *et al*., 1990 Hird *et al*., 1991 Ogihara *et al*., 2000
3	Gamma-gliadin	P08453	3.9760001[d]	7.61/37122.29	Gliadin is the major seed storage protein in wheat.	Sugiyama *et al*., 1986

4	Phosphoglycerate kinase, chloroplastic	P12782	51.88000202[*] 44.99999881[a] 35.6400013[b] 54.79000211[c] 1.875000075[e]	6.57/49839.53	Catalytic activity: ATP + 3-phospho-D-glycerate = ADP + 3-phospho-D-glyceroyl phosphate.	Longstaff *et al.*, 1989 Jones *et al.*, 1995
5	ATP synthase subunit alpha, chloroplastic	P12112	4.365000129[b] 2.380999923[c] 54.96000051[d] 50.40000081[e] 44.44000125[f]	6.11/55295.52	Produces ATP from ADP in the presence of a proton gradient across the membrane. The alpha chain is a regulatory subunit	Howe *et al.*, 1985 Ogihara *et al.*, 2000
6	Sedoheptulose-1,7-bisphosphatase, chloroplastic	P46285	3,053000011*	6.04/42060.82	Catalytic activity: Sedoheptulose 1,7-bisphosphate + H_2O = sedoheptulose 7-phosphate + phosphate.	Raines *et al.*, 1992 Miles *et al.*, 1993
7	ATP synthase subunit alpha, mitochondrial	P12862	37.92000115[d] 15.71999937[e] 18.07000041[f]	5.70/55264.20	Mitochondrial membrane ATP synthase (F(1)F(0) ATP synthase or Complex V) produces ATP from ADP in the presence of a proton gradient across the membrane which is generated by electron transport complexes of the respiratory chain. F-type ATPases consist of two structural domains, F(1) - containing the extramembraneous catalytic core, and F(0) - containing the membrane proton channel, linked	Schulte *et al.*, 1989

					together by a central stalk and a peripheral stalk. During catalysis, ATP synthesis in the catalytic domain of F(1) is coupled via a rotary mechanism of the central stalk subunits to proton translocation. Subunits alpha and beta form the catalytic core in F(1). Rotation of the central stalk against the surrounding alpha(3)beta(3) subunits leads to hydrolysis of ATP in three separate catalytic sites on the beta subunits. Subunit alpha does not bear the catalytic high-affinity ATP-binding sites (By similarity).	
8	Photosystem II CP43 reaction center protein	Q9XPS4	3.382999822[c]	6.93/52001.04	One of the components of the core complex of photosystem II (PSII). It binds chlorophyll and helps catalyze the primary light-induced photochemical processes of PSII. PSII is a light-driven water: plastoquinone oxidoreductase, using light energy to abstract electrons from H(2)O, generating O(2) and a proton gradient subsequently used for ATP formation.	Ogihara *et al*., 2000 Matsuoka *et al*., 1999
9	Fructose-1,6-bisphosphatase,	P09195	9.046000242[c]	5.14/44218.07	Catalytic activity: D-fructose 1,6-bisphosphate + H_2O = D-fructose 6-phosphate + phosphate.	Raines *et al*., 1988 Lloyd *et al*., 1991

	chloroplastic					
10	Ribulose bisphosphate carboxylase small chain PWS4.3, chloroplastic	P00871	8,620999753* 9.194999933^{a} 13.22000027^{b,c,d,e,f,}	8.99/19417.36	RuBisCO catalyzes two reactions: the carboxylation of D-ribulose 1,5-bisphosphate, the primary event in carbon dioxide fixation, as well as the oxidative fragmentation of the pentose substrate. Both reactions occur simultaneously and in competition at the same active site	Broglie *et al*., 1983 Smith *et al*., 1983 Terachi *et al*., 1987 Houtz *et al*., 1989 Mehta *et al*., 1992 Ogihara *et al*., 1991; 2000 Niu, 2003
11	Ribulose bisphosphate carboxylase small chain PW9, chloroplastic	P26667	14,2900005* 14.85999972^{a} 8.570999652^{b} 18.86000037^{c,f} 23.43000025^{d} 18.28999966^{e}	8.52/19454.45		
12	Ribulose bisphosphate carboxylase large chain	P11383	1,88699998*^{,a,b,c} 60.58999896^{d} 30.39999902^{e} 36.89999878^{f}	6.22/52851.13		
13	Ribulose bisphosphate carboxylase small	P07398	13,26999962* 14.15999979^{a} 20.35000026^{b,c}	5.84/13055.05		

	chain clone 512 (Fragment)		,d,e,f			
14	Oxygen-evolving enhancer protein 1, chloroplastic	P27665	52,31000185*	8.73/34740.37	Stabilizes the manganese cluster which is the primary site of water splitting.	Meadows *et al.*, 1991
15	ATP synthase subunit beta, chloroplastic	P20858	7.22900033[a] 9.437999874[b] 8.635000139[c] 81.52999878[d] 89.9600029[e] 75.70000291[f]	6.11/55295.52	Produces ATP from ADP in the presence of a proton gradient across the membrane. The catalytic sites are hosted primarily by the beta subunits	Howe *et al.*, 1985 Ogihara *et al.*, 2000
16	Phosphoribulokinase, chloroplastic	P26302	41.58000052[c]	5.72/45141.39	Catalytic activity: ATP + D-ribulose 5-phosphate = ADP + D-ribulose 1, 5-bisphosphate.	Raines *et al.*, 1989 Lloyd *et al.*, 1991
17	Elongation factor 1-alpha	Q03033	9.619999677[d]	9.20/49169.01	This protein promotes the GTP-dependent binding of aminoacyl-tRNA to the A-site of ribosomes during protein biosynthesis	Metz *et al.*, 1992
18	Oxygen-evolving enhancer protein 2, chloroplastic	Q00434	7,751999795*	8.84/27269.68	May be involved in the regulation of photosystem II.	James and Robbison, 1991
19	Photosystem II D2	Q36814	6,515999883*	5.34/39470.38	Photosystem II (PSII) is a light-driven water:	Hanley *et al.*, 1988

	protein		2.833000012[c] 5.38199991[e]		plastoquinone oxidoreductase that uses light energy to abstract electrons from H(2)O, generating O(2) and a proton gradient subsequently used for ATP formation. It consists of a core antenna complex that captures photons, and an electron transfer chain that converts photonic excitation into a charge separation. The D1/D2 (PsbA/PsbA) reaction center heterodimer binds P680, the primary electron donor of PSII as well as several subsequent electron acceptors. D2 is needed for assembly of a stable PSII complex.	Ogihara *et al.*, 2000
20	Photosystem II protein D1	P12463	6,232000142* 3.398999944[a,b,d,f]	5.21/38920.62		
21	2-Cys peroxiredoxin BAS1, chloroplastic (Fragment)	P80602	6.667000055[b,c]	5.71/23326.68	May be an antioxidant enzyme particularly in the developing shoot and photosynthesizing leaf.	Tsunoyama *et al.*, 1996 Tsunoyama & Toyoshima, 1997
22	RuBisCO large subunit-binding protein subunit alpha, chloroplastic (Fragment)	P08823	9.576000273[d] 9.39199999[e]	4.83/57520.59	This protein binds RuBisCO small and large subunits and is implicated in the assembly of the enzyme oligomer.	Hemmingsen *et al.*, 1988

* % coverage of proteins spot D1, [a]% coverage of proteins spot D2, [b]% coverage of proteins spot D3, [C]% coverage of proteins spot D4, [d]% coverage of proteins spot D5, [e]% coverage of proteins spot D6, [f]% coverage of proteins spot D7. 1&2 (pI/Molecular weight and function of the proteins) were obtained from www.ExPASy.org.

2.9 Summary of differentially expressed proteins by function in response to RWA SA1 feeding on both SST 347 and SST 356

Differentially expressed proteins identified by MS in the RWA SA1 resistant and moderately resistant cultivars in SST 347 and SST 356 respectively in response to stress imposed by RWA SA1 were summaried according to their functional groups (Figure 2.19).

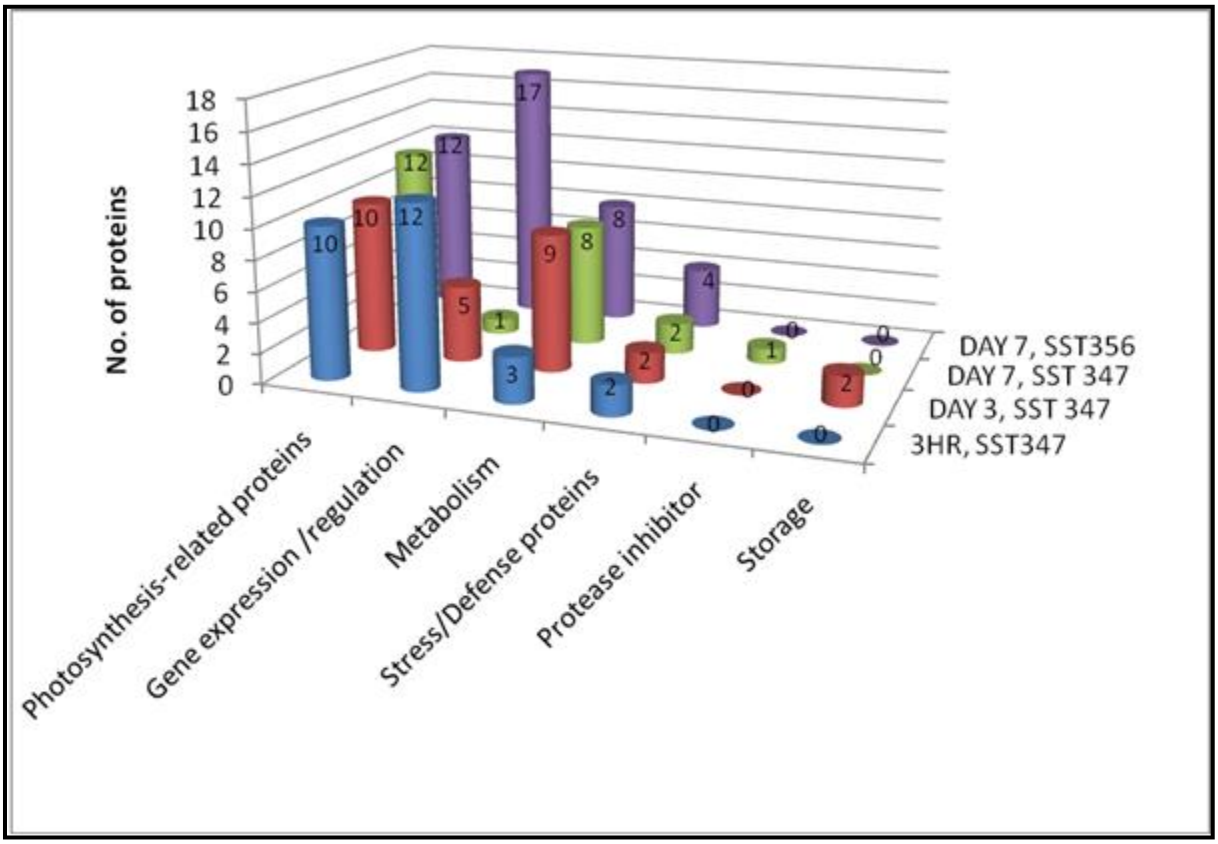


Figure 2.19: A graphical representation of differentially expressed proteins identified in both RWA SA1 infested resistant (SST 347) and moderately resistant (SST 356) wheat cultivars.

2.10 General stress-related defence responses

Plants respond to herbivory by increased transcription of many "housekeeping" genes that regulate various processes including photosynthesis, photorespiration, protein synthesis and maintenance of cell homeostasis (Karban and Baldwin, 1997). Most spots detected had keratin, trypsin and other low matching contaminants that are not discussed further. Keratin, a fibrous structural protein abundant on the outer layer of the skin of humans (*Homo sapiens*), in hairs and nails could have been introduced through sample preparation and this has often been seen as a source of contamination in MS. The other common external protein contaminant, trypsin was used in the experimental procedures for the proteolytic digestion of the proteins into peptides prior to analysis (Hogde *et al*., 2013). In all spots excised from both the resistant and the moderately resistant cultivar, a total of 63 proteins were differentially regulated and these responsive proteins to RWA infestation were grouped into six functional groups including photosynthesis related proteins, primary metabolism, gene expression and regulation, stress and defence, protease inhibitor and storage. Sixteen photosynthesis-related proteins were expressed in both the resistant and moderately resistant cultivars. Most of these proteins were found to be expressed early in the resistant cultivar (3 hrs) and only appeared later at day 7 in the moderately resistant cultivar. The proteins were mainly found in the chloroplast indicating that the resistant cultivar mounted an early response to counter chlorosis thereby delaying cell death and minimising the effect at damage incurred by the RWA SA1 (Compared these findings with other published work which found resistant cultivar delayed chlorosis)

The plant's primary metabolism was also affected since proteins involved in carbohydrate metabolism, energy metabolism, amino acids metabolism and storage proteins were upregulated. An increase in ATP synthase and other energy production related proteins indicates an enhanced demand for energy by the wheat plants to cope with the stress. Haile *et al*. (1999) stated that adjustments in photosynthetic rate in wheat can significantly contribute to its tolerance to damage/metabolic drain caused by RWA feeding. The observed up regulation of enzymes involved in photosynthesis and ATP synthesis in response to aphid infestation has been observed in rice (Wei *et al*., 2009).

Other proteins identified were stress related including oxidative stress proteins, kinases and defence related proteins. Stress/defence related proteins were up-regulated in both cultivars at 3hrs post infestation; an indication of the initiation of an early defence mechanism. The detection of oxidative stress proteins was also reported by Wei *et al*. (2009), Moloi and Van der Westhuizen, (2006, 2008) who stated that oxidative stress is one of the first general reactions to damage caused by phloem-feeding insects when they penetrate the plant. Some of these stress related proteins were also up regulated in the moderately resistant cultivar, although much later (day 7) and at a much higher level, indicating that even the moderately resistant cultivar has a defence mechanism against RWA which could be useful to minimize cereal aphid outbreaks in the field by slowing the rate of population increase. A storage protein known as gamma-gliadin was also identified in day 3 sample of the resistant cultivar.

Donnelly *et al*. (2005) also reported storage proteins such as gluten precursor in 7-10 days old seedlings and stated that this could be of interest to developmental biologists. Identified were two histone proteins (histone H2A/H2B and protein H2A) in both cultivars. They are crucial to condensed packing of DNA and are involved in transcription regulation, DNA replication and repair. Our results also indicate that some proteins were present in both cultivars such as photosystem II CP47 reaction protein, cytochrome b6-f complex iron-sulfur, oxygen-evolving enhancer protein 2, Ribulose bisphospate, ATP synthase subunit mitochondrial/chloroplastic, ATP synthase beta, sedoheptulose, 1, 7-bisphosphatase, thioredoxin and histones at 3 hrs, day 3 and day 7 post aphid infestation. These are defence related proteins expressed by the plant to cope with stress imposed by aphid feeding.

Some proteins were detected only in the resistant cultivar; cytochrome f (only in 3hrs), serpin-(Z1C, ZA1, ZA1B), ADP, ATP carrier protein 1&2 (Day 3 only), photosystem II reaction center protein, fructose-1, 6-bisphosphatase, Adenosylhomocysteinase (only in day 7), phosphoribulokinase (both day 3 and 7), while Photosystem II D2 and D1 protein, oxygen-evolving enhancer protein 1 were detected in all three timeframes. Others (profilin1, photosystem 1 non-sulfur center, cytochrome b6, cytochrome b559 subunit alpha, chlorophyll a-b binding protein, progilin 2 & 3 histone H4 variant; TH091 & TH011, ATP synthase epsilon, phosphoglycerate kinase, cytosolic, cysteine synthase and oxalate oxidase GF-3.8) were found only in moderately resistant cultivar at day 7. Those present only in the resistant cultivar may be involved in the resistance mechanism of the resistant cultivar whereas those in the moderately resistant cultivar could be a susceptibility factor, which is an indication that the plant is only moderately resistance/susceptible. Two protease inhibitor proteins (serpin-ZA1 and Serpin-Z1B) were identified only in the resistant cultivar at day 7 post aphid infestation. Habid and Fazili (2007) stated that some insects and many of the phytopathogenic microorgainsms secrete extracellur enzymes especially those causing proteolytic digestion of proteins that play an important role in pathogenesis. Plants therefore defend themselves by producing various inhibitors that act against these proteolytic enzymes. In order to identify or understand the resistant mechanism/s to RWA, the pathways were identified in which these proteins up-regulated only in the resistant cultivar are involved in.

2.10.1 Pathways identified in stress responses by wheat during RWA SA1 infestation

2.10.1.1 Methionine synthesis

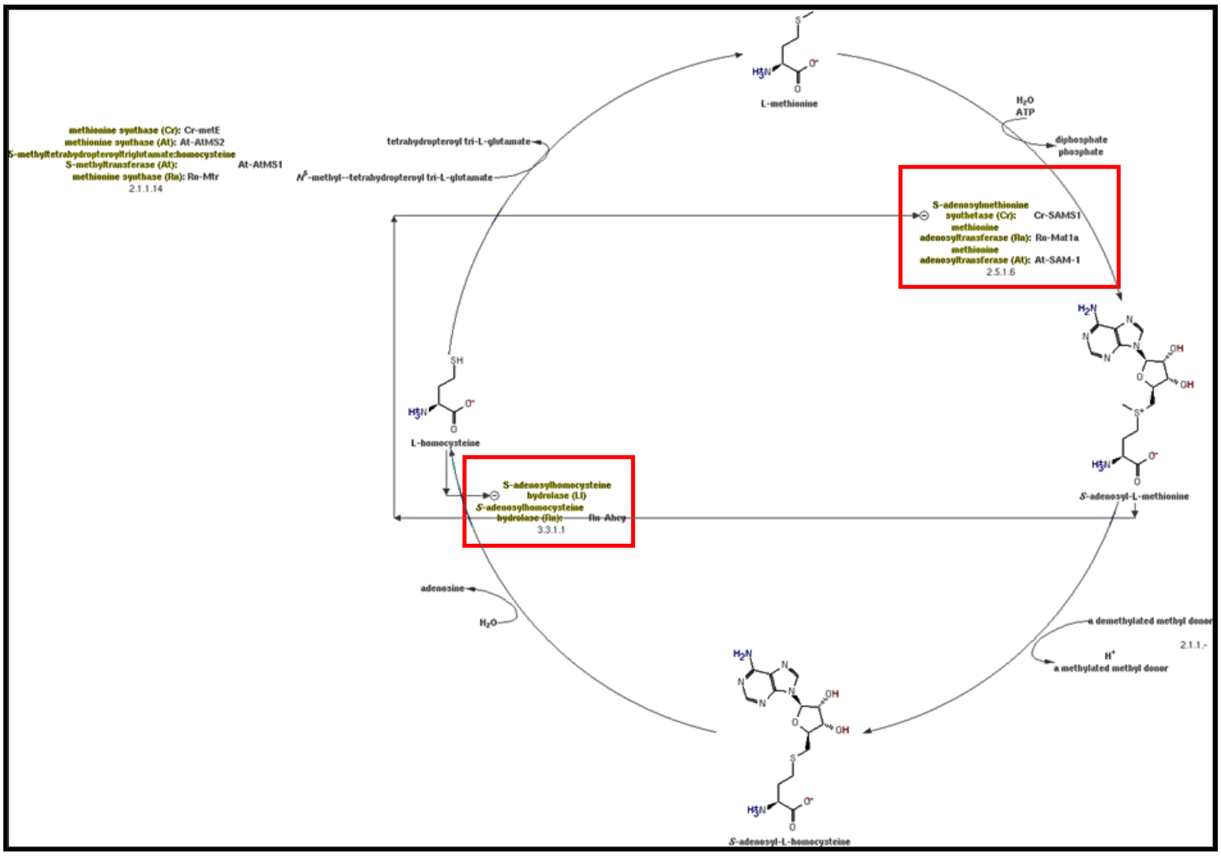


Figure 2.20: Enzymes up-regulated in the S-adenosyl-L-methionine synthesis (MetaCyc: www.metacyc.org; Caspi *et al.*, 2014) in response to aphid feeding (*Adenosylhomocysteinase, reaction S-adenosyl-L-homocysteine +* H_2O *↔ L-homocysteine + adenosine.*

S-adenosylhomocysteinase is an enzyme that catalyses the reversible hydrolysis of S-adenosyl-L-homocysteine (AdoHcy) to adenosine and L-homocysteine. The proposed mechanism of action involves the oxidation of the 3'-hydroxyl group of AdoHcy by enzyme-bound NAD^+ (Palmer and Abeles, 1979). After oxidation, L-homocysteine is eliminated, alpha-beta, to give 3'-keto-4'-5'-dehydroadenosine which reacts with water in a Michael type addition to form 3'-ketoadenosine that is then reduced to adenosine. Adenosylhomocysteinase is part of the methionine synthesis; an amino acid that does not only serve as a building block for protein synthesis but has additional roles (Ravanel *et al.*, 1998). Methionine is the immediate precursor of S-adenosylmethionine (AdoMet) that is the major methyl-group donor in transmethylation reactions and an intermediate in the biosynthesis of polyamines and the phytohormone ethylene. Up-regulation of ethylene production has been associated with early plant response to pathogen attack (Wang *et al*., 2002) which also acts in concert with jasmonic acid as a systemic signal of wound-induced gene activation by aphids (Penninckx *et al*., 1998).

2.10.1.2 Calvin cycle

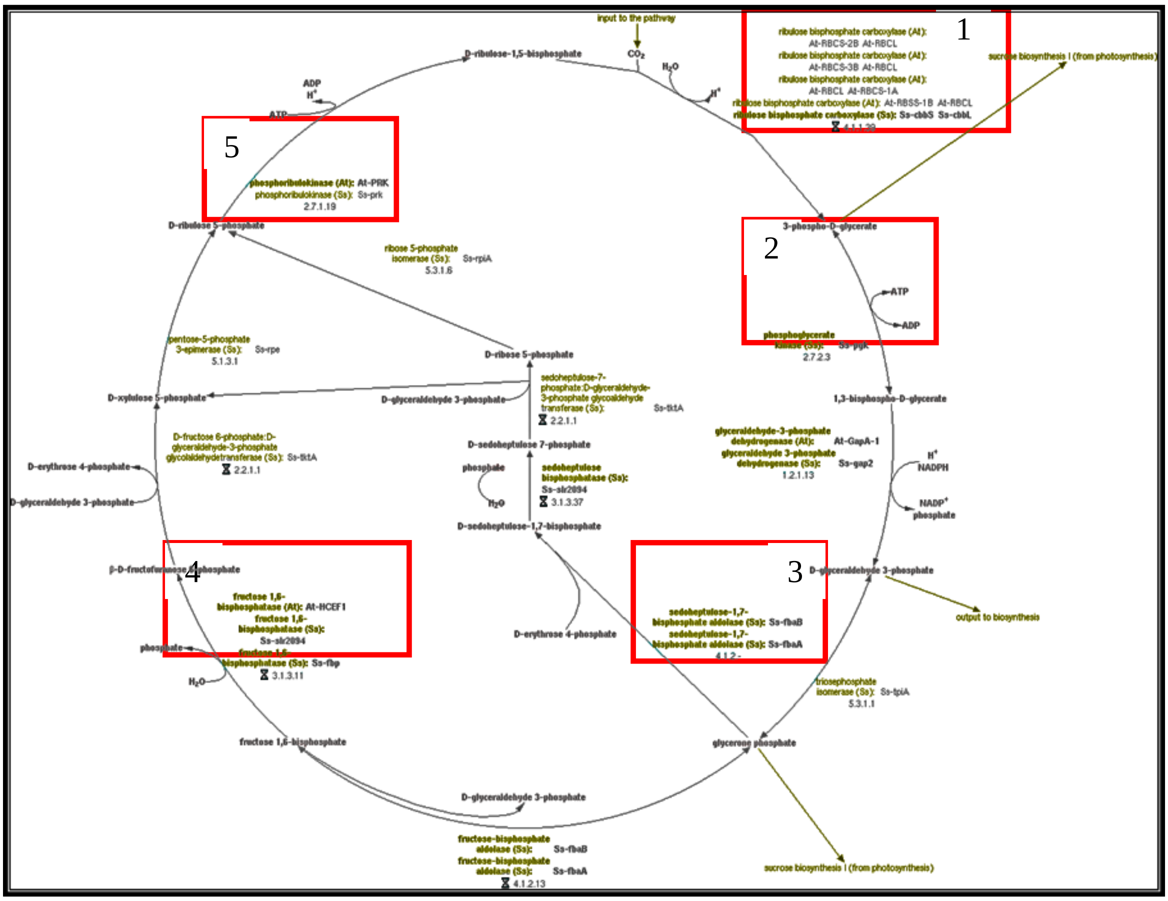


Figure 2.21: Enzymes up-regulated in the Calvin cycle (MetaCyc: www.metacyc.org; Caspi *et al*., 2014) in response to aphid feeding: 1) ribulose bisphosphate carboxylase, *reaction: 2 3-phospho-D-glycerate* + *$2H^+$* ↔ *D-ribulose-1,5-bisphosphate* + *CO_2* + *H_2O;* 2) *phosphoglycerate kinase, reaction: 3-phospho-D-glycerate* + *ATP* ↔ *1, 3-bisphospho-D-glycerate* + *ADP;* 3) *sedoheptulose-1,7-bisphosphatase, reaction: fructose 1, 6-bisphosphate* ↔ *glycerone phosphate* + *D-glyceraldehyde 3-phosphate;* 4) *fructose 1, 6-bisphospatase, reaction: fructose 1, 6-bisphosphate* + *H_2O* → *β-D-fructofuranose 6-phosphate* + *phosphate;* 5) *Phosphoribulose kinase, reaction: D-ribulose 5-phosphate* + *ATP* ↔ *D-ribulose-1, 5-bisphosphate* + *ADP* + *H^+*.

The Calvin cycle is known as the major CO_2 fixation pathway found in all green plants and many autotrophic bacteria (Caspi *et al*., 2014). This pathway is divided into three stages; fixation, reduction and regeneration involving other pathways including Phosphoribulose kinase, phosphoglycerate kinase, sedoheptulose-1, 7-bisphophatase and ribulose bisphosphate carboxylase identified in wheat response to stress imposed by aphid feeding.

In the first stage of the Calvin cycle the reduction carboxylation of D-ribulose-1, 5-bisphosphate (RuBP) s catalysed by the enzyme ribulose bisphosphate carboxylase (RubisCO) forming two molecules of 3-phospo-D-glycerate. In the second step these two molecules are phosphorylated to 1, 3-bisphospho-D-glycerate and then reductively dephosphorylated to D-glyceraldehyde 3-phosphate. In three turns of the cycle 3 molecules of CO_2 are fixed and 6 molecules of D-glyceraldehyde 3-phosphate are formed of which one is diverted to biosynthetic pathways while the other 5 are used up in the next stage. The third stage comprised of a series of rearrangements that would eventually regenerate RuBP. Some of the D-glyceraldehyde 3-phosphate are converted to glycerone phosphate. D-glyceraldehyde 3-phosphate and glycerone phosphate are then condensed into fructose 1, 6-bisphospate which is dephosphorylated to β-D-fructose 6-phosphate. The later combines with another D-glyceraldehyde 3-phosphate molecule and cleaved into D-xylulose 5-phosphate (X5P) and D-erythrose 4-phosphate. D-erythrose 4-phosphate combines with glycerine phosphate to form D-sedoheptulose-1, 7-bisphosphate, that is dephosphorylated to D-sedoheptulose 7-phosphate. This combines with D-glyceraldehyde 3-phosphate and is cleaved into a second D-xylulose 5-phosphate and a D-ribose 5-phosphate (R5P). Both of these are converted into D-ribulose 5-phosphate that is finally phosphorylated to D-ribulose-1, 5-bisphosphate, regenerating the key CO_2-acceptor molecule (Caspi *et al*., 2014)

In this cycle especially during the day 3-phospo-D-glycerate, glycerine phosphate or D-glyceraldehyde 3-phosphate can be siphoned off and sent out to the cytosol via the triose phosphate transporter for sucrose biosynthesis I (from photosynthesis). Plants need to carefully control the carbon flux between this and competing pathways to make sure that the Calvin cycle can proceed (Caspi *et al*., 2014). To achieve this, the catalytic activities of certain enzymes within the cycle are highly regulated including sedoheptulose-1, 7-bisphosphatase (SBPase) and fructose-1, 6-bisphosphatase (FBPase) by the redox potential via the ferredoxin/thioredoxin system that modulates the enzyme activities in response to light/dark conditions (Scheibe, 1990; Buchanan, 1991). Also, FBPase functions at the branch point between the regenerative phase of the Calvin cycle and starch biosynthesis, and SBPase and FBPase catalyse irreversible reactions (KoBmann *et al*., 1994). Lefebvre *et al*. (2005) reported that increase in SBPase stimulates photosynthesis and growth rate in Arabidopsis. Increases in the enzymes involved in the plant's primary metabolism in response to aphid could be a means by which the plant is adapting to the damage imposed by the RWA or to the leakage/loss of metabolites as a result of aphid feeding.

2.10.1.3 Cysteine biosynthesis

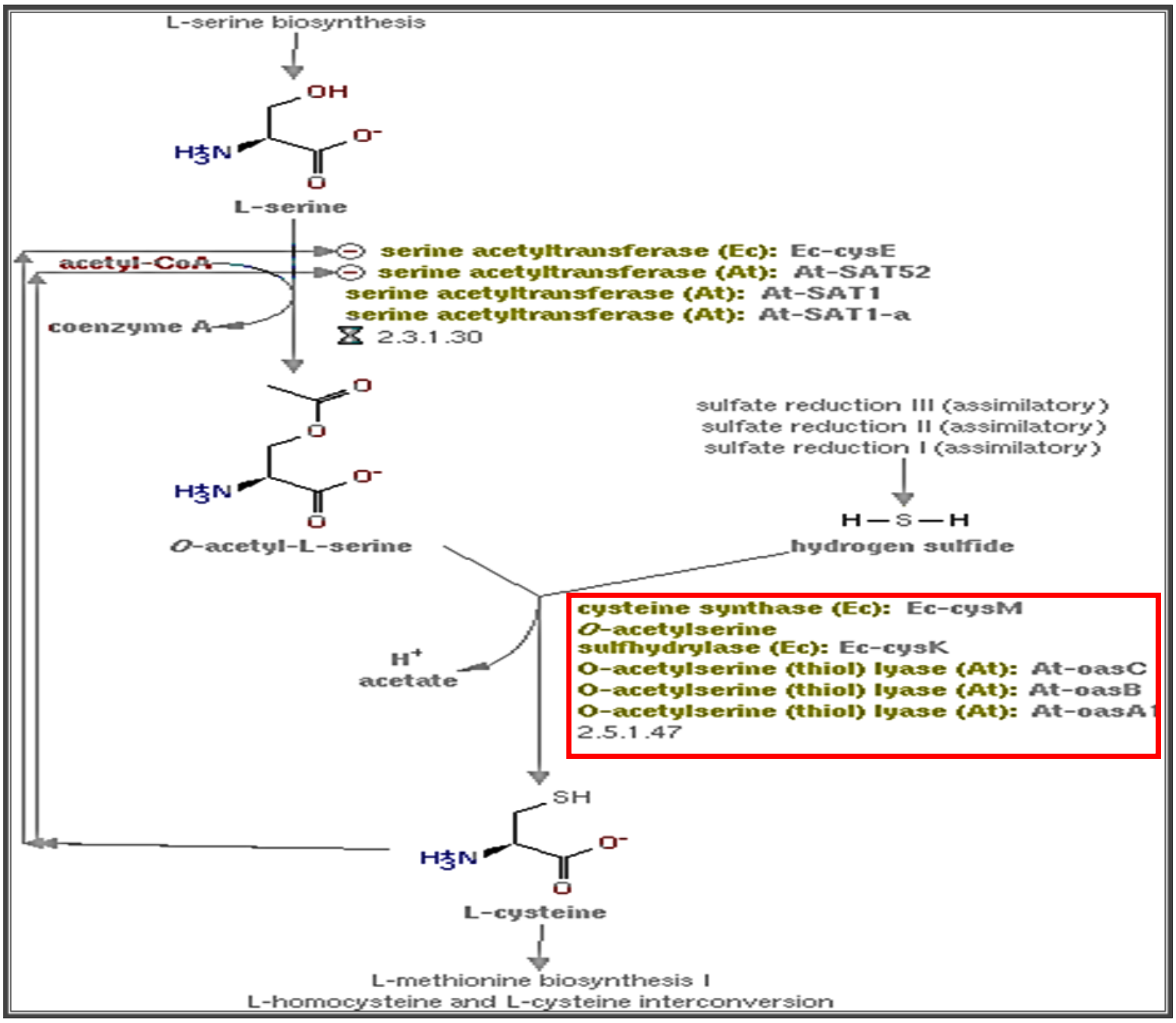


Figure 2.22: Enzyme up-regulated in the cysteine biosynthesis pathway (MetaCyc: www.metacyc.org; Caspi *et al.*, 2014) in response to aphid feeding: Cysteine synthase, *reaction, L-serine + acetyl CoA + sulfide = L-cysteine + CoA + acetate.*

This pathway involved in the biosynthesis of cysteine is a two-step conversion of L-serine to L-cysteine via O-acetyl-L-serine. This pathway has been documented in many organisms including bacteria (Kredich, 1996), archaea (Kitabatake *et al*., 2000) and plants (Jost *et al*., 2000). Cysteine synthase is a key enzyme in this pathway and its product; cysteine is one of the compounds known to be involved in plant defence systems against toxic heavy metal. The synthesis of this molecule is vital in plant defence. An up regulation of cysteine synthase in elevated concentrations of aluminium was reported in rice by Yang *et al*. (2007). This enzyme is involved in glutathionine metabolism pathway. Although the enzyme has been associated with abiotic stress, our results indicate that cysteine synthase is involved in stress imposed (wounding) by aphid feeding and thus further investigation is needed to associate this enzyme to biotic stress.

2.10.1.4 ATP synthesis

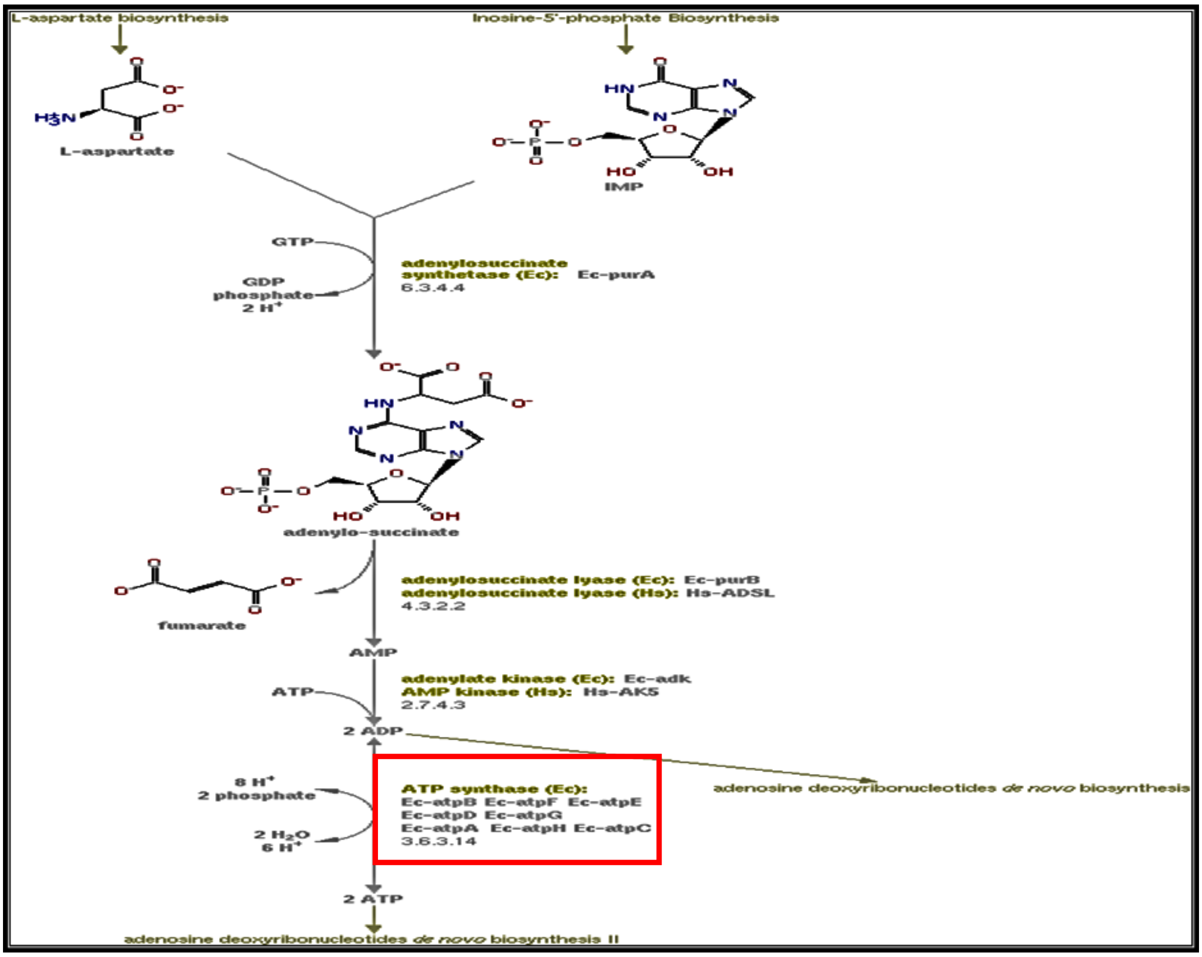


Figure 2.23: Enzyme up-regulated in the ATP synthesis pathway (MetaCyc: www.metacyc.org; Caspi *et al.*, 2014) in response to aphid feeding: ATP synthase, *reaction, transport of* H^+.

ATP synthase (F0F1 synthase) catalyses the synthesis of ATP from ADP and inorganic phosphate (Pi). This enzyme has two sub complexes known as F_1 and F_0. The hydrophilic F_1 complex consists of five subunits (α, β, γ, δ and ε) occurring in the ratio of 3:3:1:1:1 with 3 catalytic sites located in the three α/β subunit pairs. The F_0 complex is membrane-embedded and forms the proton channel through the membrane with 3 subunits (a, b and c) in the ratio of 1:2:10. ATP is known to induce the expression of genes involved in the biosynthesis of jasmonic acid and ethylene which important pathways in plant defence (Choi *et al.*, 2014). JA and ET have been postulated to activate specific genes that promote wound healing (Asahina *et al.*, 2011).

2.10.1.5 Thioredoxin pathway

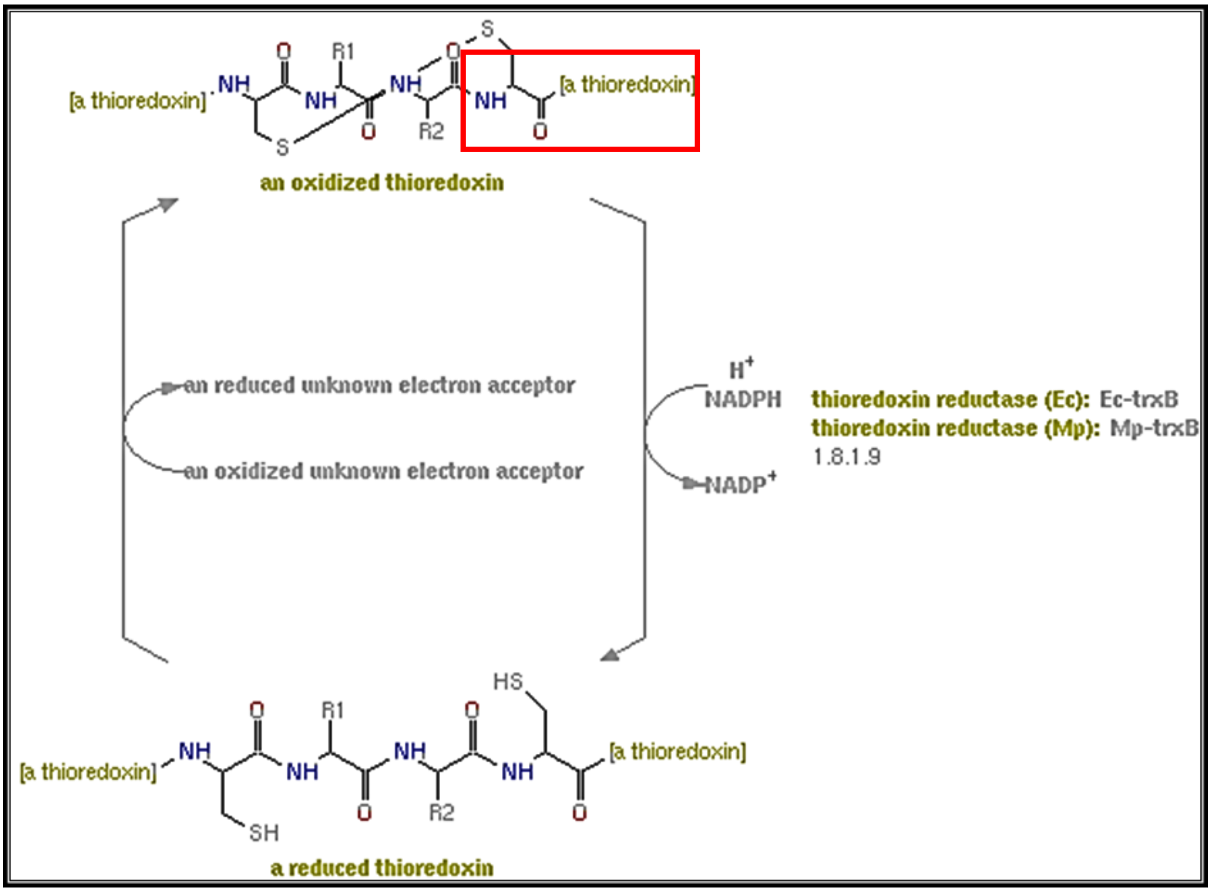


Figure 2.24: Enzyme up-regulated in the Thioredoxin pathway (MetaCyc: www.metacyc.org; Caspi *et al.*, 2014) in response to aphid feeding.

Thioredoxins (TRXs) are redox proteins involved in regulation of numerous target proteins via thiol/disulfide exchanges. They therefore play key roles in the maintenance of cellular redox homeostasis through the sensing and transfer of reducing equivalents (Eckardt, 2007). It is increasingly recognised that TRXs play an important role as a signalling intermediate that senses the redox state and transmits this information to other signalling molecules in chloroplasts, mitochondria, and the cytosol (Fujino *et al*., 2006). Redox signalling within the chloroplast has long been recognised as a key component of photosynthesis, and chloroplast TRXs are known to regulate numerous photosynthetic enzymes such as glyceraldehyde 3-phosphate dehydrogenase, fructose 1, 6-bis phosphatase, sedoheptulose 1, 7-bis phosphatase, phosphoribulokinase, and rubisco activase (Hisabori *et al*., 2007). The up-regualtion of thioredoxin is evident that the salicylic acid pathway in response to pathogen attack has been activated see Figure 1.5, section 1.6.1. Therefore its role in wheat resistance to Russian wheat aphid feeding has to be further investigated.

2.10.1.6 Oxalate oxidase pathway

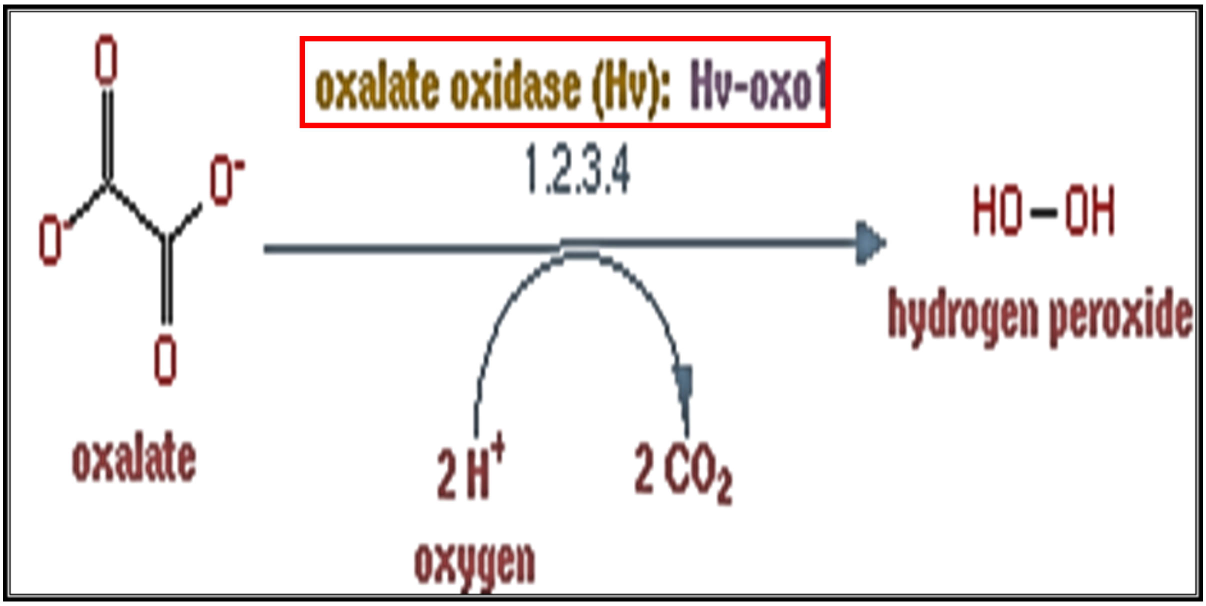


Figure 2.25: Enzyme up-regulated in the oxidative cleavage of CO_2 (MetaCyc: www.metacyc.org; Caspi *et al*., 2014) in response to aphid feeding: Oxalate oxidase, reaction, *oxalate + oxygen + 2* $H^+ \rightarrow 2CO_2$ *+ hydrogen peroxide.*

Oxalate oxidase is an enzyme that catalyses the oxidative cleavage of carbon dioxide with reduction of dioxygen to hydrogen peroxide. Although widespread in cereal plants, it has also been reported in bacteria and fungi (Dumas *et al*., 1995). The enzyme is involved in the defence against biotic and abiotic stress in plants. Enzyme activity has been detected in barley seedling roots during growth and development and in the leaves of mature plants in response to powdery-mildew infection (Dumas *et al*., 1995). Nemati *et al*. (2005) reported that oxalate oxidase defended wheat plants from *Fusarium graminearum*. In another study by Jie and Makot (2004), this enzyme was found in rice defence systems against rice blast. Although this enzyme has been reported to protect plants against infection, the role of this enzyme in defending plants against aphid infestation is not known. The exact nature of the elevated resistance to pathogens is still not clear; it has however been postulated that it acts by generating microcidal concentrations of hydrogen peroxide, eliciting hypersensitive cell death at lower concentrations of H_2O_2, forming effective barriers against penetration by the hydrogen-peroxide-mediated lignification of cell walls and destruction of oxalate, an inhibitor of the hypersensitive response. Therefore it is important to further investigate the role of oxalate oxidase in wheat response to aphid infestation.

2.11 Conclusion

Proteomic analysis showed that stress response proteins were up-regulated in resistant diploid wheat (*Triticum aestivum)* cultivar SST 347 in response to attack by the phloem feeding aphid (*Duiraphis noxia*). Garcia-Burgger *et al*. (2006) also reported early plant responses to aphid and/or pathogen infections which shares common events such as phosphorylation, membrane depolarization, calcium influx and release of reactive oxygen species (ROS) leading to the activation of phytohormone-dependent pathways. Ethylene and jasmonate dependent responses are activated by insects (Maffie *et al*., 2007). Other pathways identified in the stress response were more related to sucrose and energy metabolism. Previous studies (Ibraheem *et al*., 2008; 2011; 2013) have identified a key role for Sucrose Transporter in the stress response to aphid infection, mainly as a result of loss of assimilates due to phloem damage. Generally most of the pathways identified were involved in the resistance mechanism of the wheat plant, were involved in maintaining the integrity of the chloroplasts and the photosynthetic systems. Therefore of interest in future would be to study the proteome of the chloroplasts.

CHAPTER THREE

MECHANISM OF LEAF RUST RESISTANCE IN WHEAT CULTIVARS

3.1 Introduction

Rust fungi are devastating plant pathogens and several *Puccinia* species have a large economic impact on wheat production worldwide with significant annual decrease in yields recorded (Webb and Fellers, 2006; Panvar *et al.*, 2013). *Puccinia triticina* (pt), otherwise known as leaf rust, is the causative agent of Wheat (*Triticum aestivum* L. and *T.durum*) leaf rust disease and is one of the most destructive diseases of wheat (Bolton *et al*., 2008). In the Republic of South Africa, leaf rust race 3SA145 was first reported in 2009 with virulence for the adult plant resistance gene *Lr*37 (Terefe *et al*., 2014). The disease is more frequent in the winter rainfall regions of the Western Cape and areas where wheat is grown under irrigation (Terefe *et al*., 2009) with susceptible cultivars suffering more than 50% yield loss (Boshoff *et al*., 2002). Management of leaf rust is often achieved by growing resistant cultivars and/or by application of fungicides. However, resistant cultivars are preferred over the intensive use of fungicides (Pretorius *et al*., 2007; Huerta-Espino *et al*., 2011), although some constraints do exist in using resistant cultivars to control leaf rust. The most challenging of this genetic control being that Pt frequently acquires new virulence to overcome effective resistance in existing cultivars, in particular when resistance is conferred by race specific genes (Huerta-Espino *et al*., 2011; Terefe *et al*., 2011; Panwar *et al*., 2013).

Extensive genomic studies have identified vital pathogenicity genes denoted as *Lr*, however these studies were hindered due to the biotrophic life style of the rust fungi. Since proteins are directly involved in plant stress responses (Kosova *et al*., 2014), it is imperative to study the proteome changes of wheat during leaf rust infection so as to clarify the molecular details and components of the resistance response in wheat to combat leaf rust. Generally, both abiotic and biotic stressors are known to induce profound alterations in various protein networks covering signalling, energy metabolism (glycolysis, Krebs cycle, ATP biosynthesis and Photosynthesis), storage proteins, protein metabolism and several other biosynthetic pathways (such as S-adenosylmethionine metabolism, lignin metabolism), transport proteins, proteins involved in protein folding and chaperone activities, other protective proteins (LEA, PR proteins), ROS scavenging enzymes as well as proteins affecting regulation of plant growth and development (Mehta *et al.*, 2008; Kosova *et al.*, 2014)

Plant-pathogen interaction has been described as a multifaceted process with plants developing two pathways to recognise and resist pathogen attack at the very beginning of the interaction (De Wit, 2007; Dodds and Rathjen, 2010). One of the pathways involves the formation of danger-association molecular patterns (DAMPs) and pathogen associated molecular patterns (PAMPs). After recognition of the pathogen by specific pathogen effector molecules, the second pathway is triggered which results in effector-triggerred immunity (ETI) and PAMPs-triggered immunity (PTI) (De Wit, 2007; Dodds and Rathjen, 2010; Lodha and Basak, 2012). Numerous proteins such as bacterial flagellin, lipopolysaccharides (LPs), and elongation factor Tu(EF-Tu), fungal chitin and oomycetes cellular-binding eliciting proteins (Zipfel, 2008; Schwessinger and Ronald, 2012) as well as signalling pathways including Salicyclic acid, Jasmonic acid and ethylene are involved in this precisely controlled multifaceted process (Glazebrook, 2005; Vlot *et al.*, 2009; Derksen *et al.*, 2013). Conventional biochemical and genetic experimental approaches are insufficient to explain the intricacy of plant-pathogen interactions (Lodha and Basak, 2012). Therefore, in recent years proteomics has played a key role in identifying changes at the protein level in plant hosts during infection by the pathogenic organisms and in characterising cellular and extracellular virulence and pathogenicity factors produced by pathogens (Rampitch *et al.*, 2006; Lohha *et al.*, 2013).

Two-Dimensional Electrophoresis (2-DE), is one of the most reproducible, precise and efficient method of separating complex proteins. It is a powerful tool employed in proteomics for purification and characterization of proteins (O’Farrel, 1975; Rampitsch *et al.*, 2006); 2-DE exploits two independent properties; Isoelectric point PI and molecular size of proteins to separate particular protein from a mixture of proteins (Klose, 1975). Isoelectric focusing separates the proteins according to the charge present on them in a first direction and for the second direction; sodium dodecyl sulfate (SDS) PAGE is used to separate proteins according to their size (Gorg *et al.*, 2000). The advantage of this technique is that it provides a good platform to analyse a mixture of proteins and up to 5000 protein spots can be identified on a large format of 2-DE-gel. Separated protein spots are visualised by staining the gel with different staining techniques such as coomassie blue staining, silver staining or SYPRO staining (Chevalier, 2010). SYPRO is a collection of molecular probe for protein detection and stains for luminescent detection of proteins separated by PAGE. Digital images of 2-DE gels are captured by sensitive scanners and analysed using soft wares such as Melanie, PDQuest, Phoretix, Progenesis, Z3 or Z4000. Spots of interest are excised either manually or robotically from the gel, digested with sequence specific proteases and the identity of proteins analysed by mass spectrometry 16, 17, 18, 7, 9 (Lodha *et al*., 2013). Most of the proteomic studies investigating plant stress are comparative studies that are based on comparison of proteome composition in stressed plants versus their unstressed (control) counterparts (Kosova *et al*., 2014). The aim of this study was therefore to explain the resistant mechanism of wheat cultivars to leaf rust infection by determining differentially expressed proteins in a leaf rust resistant cultivar (SST 347) comparing it to a susceptible cultivar (SST 356).

3.2 Materials and methods

Seeds of two wheat cultivars, SST 347 (Resistant to RWA SA1 and leaf rust race 3SA145) and SST 356 (Moderately Resistant to RWA SA1 and susceptible to the leaf rust race) were obtained from SENSAKO, Bethlehem while leaf rust race 3SA145 spores and the susceptible Morocco wheat line were obtained from the Agricultural Research Council of South Africa (Bethlehem, South Africa). Hydromix and hygrofert were purchased from Hygrotech East London, SA. The total protein extraction, protein quantification (RCDC) kits, 2-DE clean up kits, 2-DE starter kits, IPG strips, rehydration trays, criterion gels (14%), sypro ruby protein stain and PDQuest software were procured from Bio-Rad (U.S.A.). Liquid nitrogen was obtained from the Department of Chemistry, Rhodes University, Grahamstown, South Africa.

3.2.1 Revival of Fungal spores

The spores were dissolved in soltrol 170 in a spraying bottle and spore-soltrol suspension used to spray the plants. The plants were placed under the light to allow the oil to dry completely and thereafter the leaves were sprayed with sterile distilled water. Plants were then transferred to a dew chamber at 30-35 ºC overnight for the fungus to infect the plants. After the overnight incubations, plants were taken out and allowed to dry again. When completely dried, they were moved to a glasshouse at 22-23 ºC. As the spores germinated they were harvested and used immediately to infect experimental plants with the left overs stored in sterile vials at -80 ºC.

3.2.2 Experimental Design

Host plant material were grown and maintained in a conviron as explained in section 2.2.3. A total of 372 plants (186 SST 347 and 186 SST 356) (Figure 3.1) were grown to 3-4 leaf stage with half of each as tests (infected with leaf rust) and the other half as controls (uninfected). Fresh spores were suspended in light mineral oil (Soltrol 170) sprayed on the test plants while the control plants were sprayed with Soltrol 170 only. All the plants were placed and dried under light for 5 minutes. The plants were then transferred to the dew chamber sprayed with distilled water and incubated overnight at 18 °C. After the overnight incubation, plants were removed from the dew chamber and again dried under light for 2-3 hrs. After drying, the plants were transferred to glass house set at 22 °C day and 23 °C night. Plants in each pot were pooled together with 2 leaves harvested from each plant at various time frames following inoculation and 0 hr (before inoculation). Leaves were harvested from both the test and control simultaneously.The leaves were placed in 15 mL falcon tubes and immediately frozen in liquid nitrogen and stored at -80 °C.

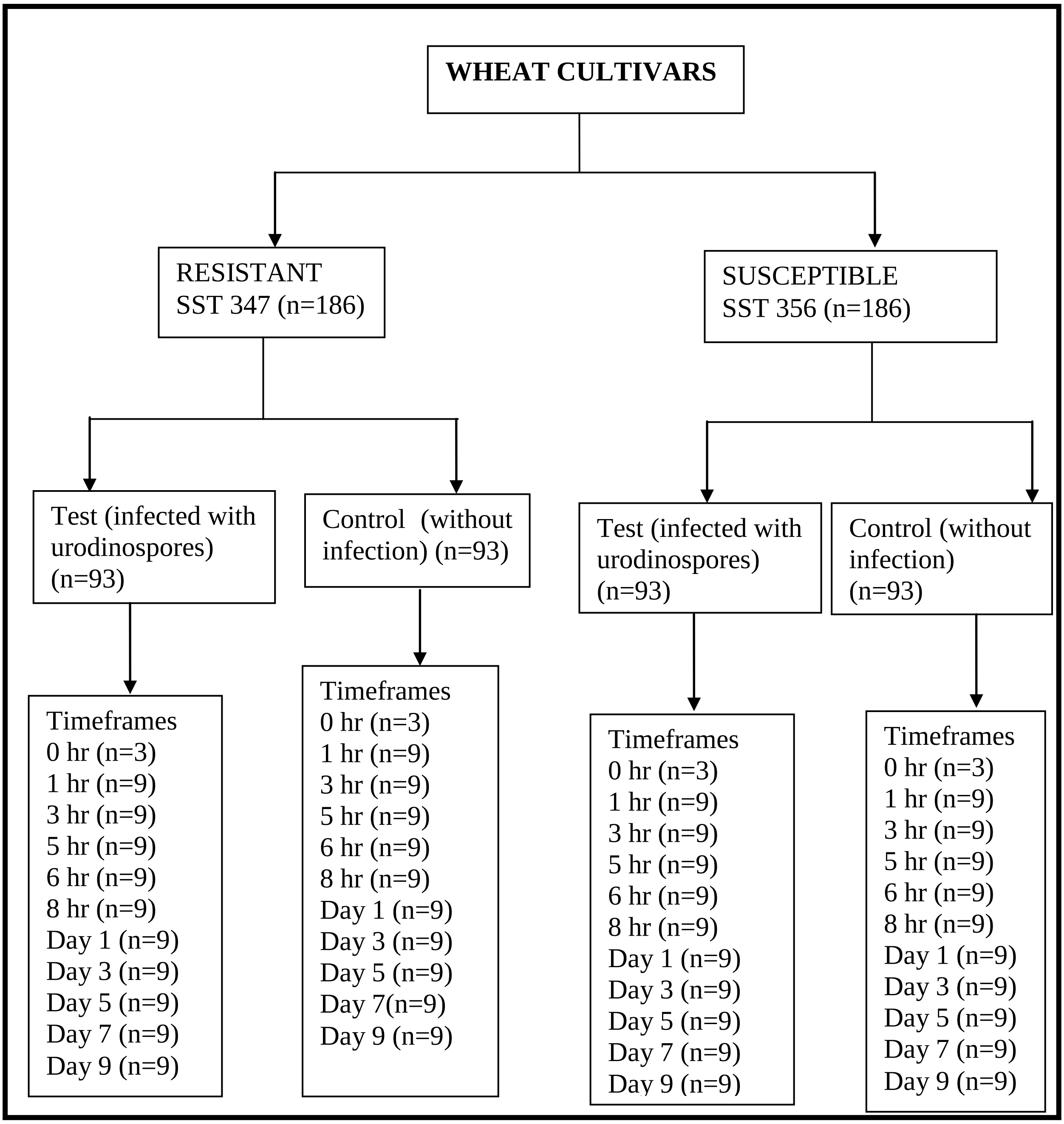


Figure 3.1: Schematic presentation of wheat cultivars used (n=number of plants) in the leaf rust experiment

3.2.4 Identification of differentially regulated proteins

In order to identify proteins that were differentially regulated in the wheat cultivars in response to leaf rust infection, all proteomic based experiments were performed as earlier outlined in section 2.2.6 to section 2.2.14.6

3.5 Results

3.3.1 Differentially expressed proteins at 3 hrs following leaf rust infection

The proteome of both the resistant and susceptible wheat cultivars at 3 hrs after infection was the same (Figure 3.2A & B).

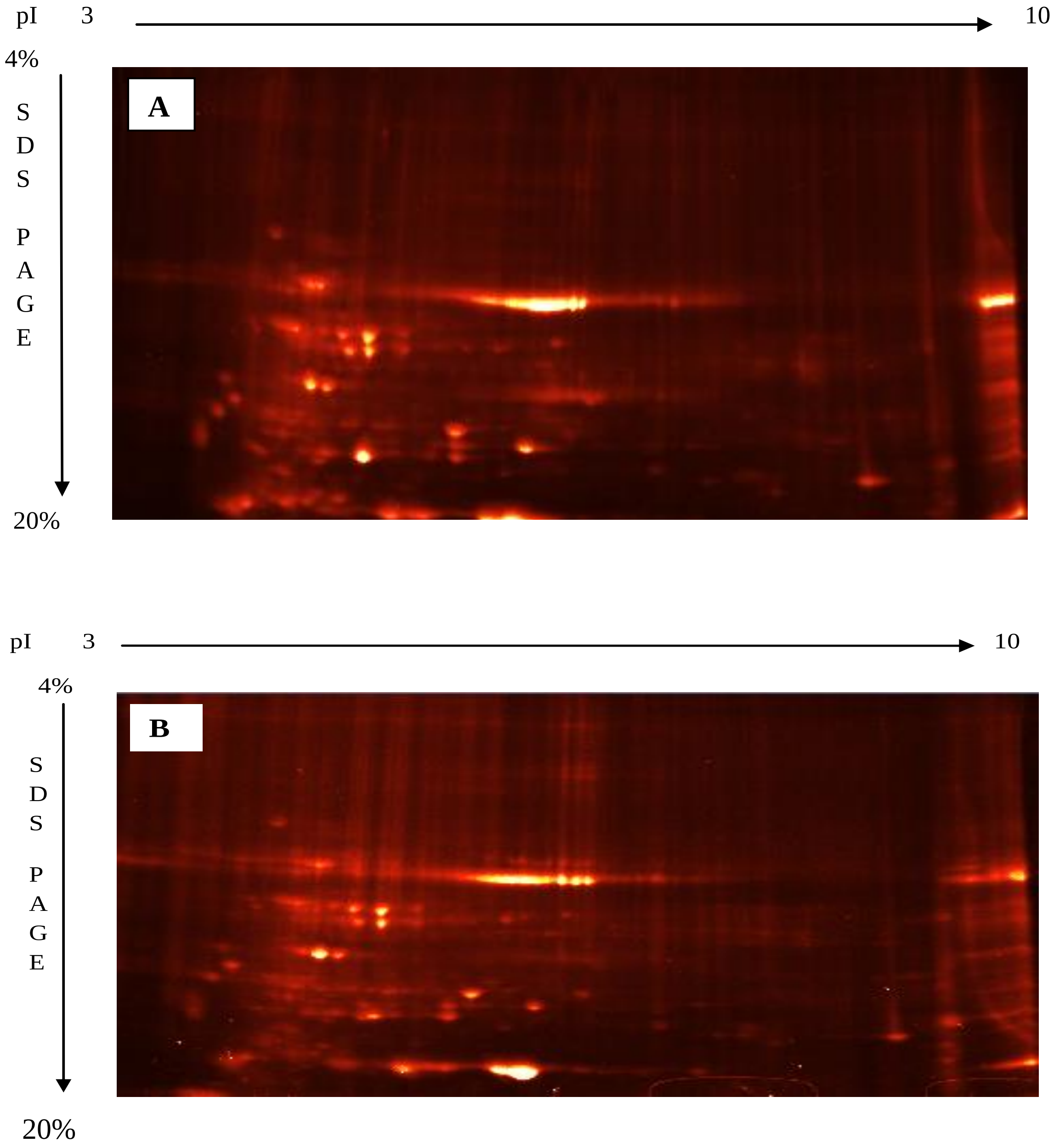


Figure 3.2: 2-D gel of SST 347 (A) & SST 356 (B) at 3hrs after leaf rust infection; no up-regulation.

3.3.2 Differentially expressed proteins at day 3 following leaf rust infection

The gels of both wheat cultivars were compared with their respective controls Day 3 after infection and there was no difference in the spots identified by 2-DE. However, comparing the test gels of SST 347 to that of SS7 356, 3 protein spots appeared on the SST 347 gels (Figure 3.3). A total of 21 proteins were identified from the 3 spots (E1, E2 and E3) that were deferentially regulated at Day 3 following leaf rust infection. The following proteins were most likely to represent the spots; Phosphoglycerate kinase (E1), ATP synthase subunit beta (E2) and Ribulose bisphosphate carboxylase small chain clone (E3). The other proteins identified must have co-migrated with these ones (Table 3.1).

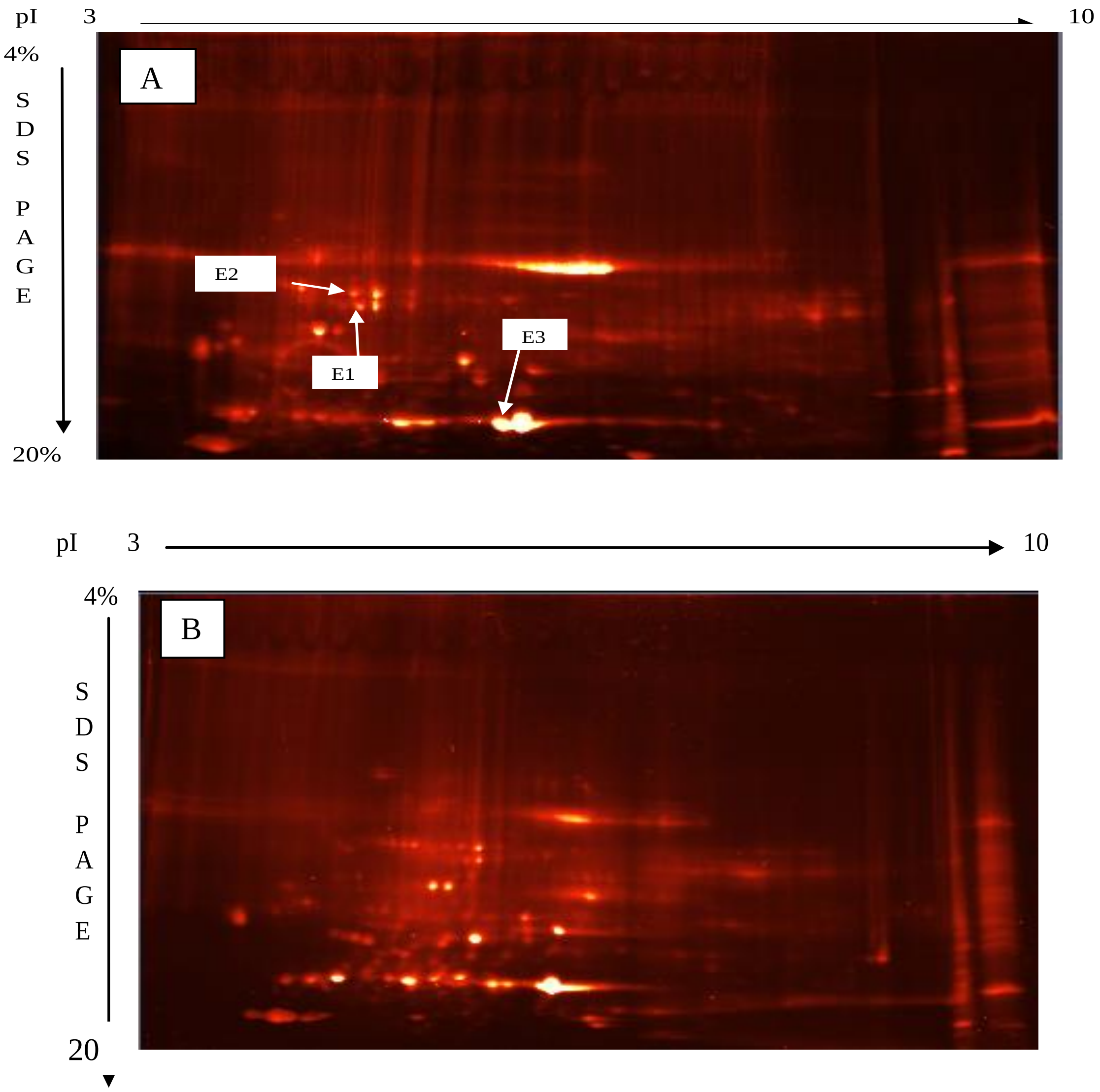


Figure 3.3: 2-D gel of SST 347 (A) Day 3 after leaf rust infection, E1-E3, up-regulated protein spots when compared to SST 356 (B) excised from the gel.

Table 3.1: Proteins identified from the wheat leaf proteome (SST 347) on day 3 after leaf rust infection

N	Protein (s) identified	Accession number	% coverage (95%)	pI /Molecular weight (Dalton)^{1}	Function^{2}	References
1	Cytochrome b6-f complex iron-sulfur subunit, chloroplastic	Q7X9A6	11.71000004^{b}	8.47/23726.26	Component of the cytochrome b6-f complex, which mediates electron transfer between photosystem II (PSII) and photosystem I (PSI), cyclic electron flow around PSI, and state transitions	Hird *et al*., 1991 Ogihara *et al*., 2000 Lu *et al*., 2002
2	Cytochrome b6	P60162	9.766999632^{a}	8.98/24182.58		
3	Oxygen-evolving enhancer protein 2, chloroplastic	Q00434	3.875999898* 22.48000056^{a} 17.04999954^{b}	8.84/27269.68	May be involved in the regulation of photosystem II.	James and Robinson, 1991
4	Phosphoglycerate kinase, chloroplastic	P12782	74.79000092* 68.33000183^{a} 9.375^{b}	6.57/49839.53	Catalytic activity: ATP + 3-phospho-D-glycerate = ADP + 3-phospho-D-glyceroyl phosphate.	Longstaff *et al*., 1989 Jones *et al*., 1995
5	S-adenosylmethionine synthase	B0LXM0	3.787999973*	5.55/43179.90	Catalyzes the formation of S-adenosylmethionine from methionine and ATP. The overall synthetic reaction is composed of two sequential steps, AdoMet formation and the subsequent tripolyphosphate hydrolysis	Wang *et al*., 2008

					which occurs prior to release of AdoMet from the enzyme (By similarity).	
6	Cysteine synthase	P38076	12.30999976	5.46/34114.38	Catalytic activity: O-acetyl-L-serine + hydrogen sulfide = L-cysteine + acetate.	Youssefian *et al*., 1993
7	Photosystem II protein D1	P12463	11.90000027* 15.00999928[a]	5.21/38920.62	Photosystem II (PSII) is a light-driven water: plastoquinone oxidoreductase that uses light energy to abstract electrons from H(2)O, generating O(2) and a proton gradient subsequently used for ATP formation. It consists of a core antenna complex that captures photons, and an electron transfer chain that converts photonic excitation into a charge separation. The D1/D2 (PsbA/PsbA) reaction center heterodimer binds P680, the primary electron donor of PSII as well as several subsequent electron acceptors.	Hanley-Bowdoin & Chua, 1998 Ogihara *et al*., 2000
8	Photosystem II D2 protein	Q36814	9.064999968*,[a]	5.34/39470.38		
9	Photosystem II CP43 reaction center protein	Q9XPS4	9.724999964* 5.496999994[a]	6.93/52001.04	One of the components of the core complex of photosystem II (PSII). It binds chlorophyll and helps catalyze the primary light-induced	Matsuoka *et al*., 1999 Ogihara *et al*., 2000

10	Photosystem II CP47 reaction center protein	P24065	2.164999954*	6.06/56092.03	photochemical processes of PSII. PSII is a light-driven water: plastoquinone oxidoreductase, using light energy to abstract electrons from H(2)O, generating O(2) and a proton gradient subsequently used for ATP formation.	
11	Phosphoglycerate kinase, cytosolic	P12783	16.9599995* 19.20000017[a]	5.64/42121.96	Catalytic activity: ATP + 3-phospho-D-glycerate = ADP + 3-phospho-D-glyceroyl phosphate.	Longstaff *et al.*, 1989
12	Phosphoribulokinase, chloroplastic	P26302	41.08999968* 38.37000132[a]	5.72/45141.39	Catalytic activity: ATP + D-ribulose 5-phosphate = ADP + D-ribulose 1, 5-bisphosphate.	Raines *et al.*, 1989 Lloyd *et al.*, 1991
13	Sedoheptulose-1,7-bisphosphatase, chloroplastic	P46285	37.40000129* 41.98000133[a\] 3.562000021[b]	6.04/42060.82	Catalytic activity: Sedoheptulose 1, 7-bisphosphate + H_2O = sedoheptulose 7-phosphate + phosphate.	Raines *et al.*, 1992 Miles *et al.*, 1993
14	ATP synthase subunit beta, chloroplastic	P20858	67.47000217* 73.48999977[a] 8.8349998[b]	5.06/53857048	Produces ATP from ADP in the presence of a proton gradient across the membrane. The catalytic sites are hosted primarily by the beta subunits.	Howe *et al.*, 1985 Ogihara *et al.*, 1991; 2000
15	ATP synthase subunit alpha, chloroplastic	P12112	27.77999938* 24.60000068[a]	6.11/55295.52		

16	Ribulose bisphosphate carboxylase small chain clone 512	P07398	99.11999702[b]	5.84/13055.05	RuBisCO catalyzes two reactions: the carboxylation of D-ribulose 1,5-bisphosphate, the primary event in carbon dioxide fixation, as well as the oxidative fragmentation of the pentose substrate. Both reactions occur simultaneously and in competition at the same active site (By similarity).	Broglie *et al*., 1983 Smith *et al*., 1983 Terachi *et al*., 1987 Houtz *et al*., 1989 Mehta *et al*., 1992 Ogihara *et al*., 1991; 2000 Niu, 2003
17	Ribulose bisphosphate carboxylase large chain	P11383	23.05999994* 22.01000005[a] 17.3999995[b]	6.22/52851.13		
18	Ribulose bisphosphate carboxylase small chain PW9, chloroplastic	P26667	18.86000037* 31.43000007[a] 73.71000051	8.52/19454.45		
19	Ribulose bisphosphate carboxylase small chain PWS4.3, chloroplastic	P00871	13.22000027* 21.25999928[a] 74.14000034[b]	8.99/19417.36		

21	Chlorophyll a-b binding protein, chloroplastic	P04784	13.16000074[a]	5.67/28264.35	The light-harvesting complex (LHC) functions as a light receptor, it captures and delivers excitation energy to photosystems with which it is closely associated.	Lamppa *et al*., 1985
20	2-Cys peroxiredoxin BAS1, chloroplastic (Fragment)	P80602	6.667000055[*, a]	5.71/23326.68	May be an antioxidant enzyme particularly in the developing shoot and photosynthesizing leaf.	Tsunoyama *et al*., 1996 Tsunoyama & Toyoshima, 1997

[*] % coverage of proteins in spot E1; [a] % coverage of proteins in spot E2; [b] % coverage of proteins in spot E3. 1&2 (pI/Molecular weight and function of the proteins) were obtained from www.ExPASy.org.

3.3.3 Differentially expressed proteins at Day 7 following wheat Leaf rust infection

There was no difference in proteins expressed between the test and the respective controls of SST 356 and SST 347. However, 3 protein spots were present on the test gels of SST 356 and absent in SST 347 test (Figure 3.4B). On the other hand, 6 protein spots were present in SST 347 and absent in SST 356. From the 3 spots (F1, F2, F3), 25 proteins were identified while 30 proteins were identified from the 6 spots (G1, G2, G3, G4, G5, G6) (Figure 3.4A). The most likely proteins representing the spots includes Ribulose bisphosphate carboxylase small chain clone 512 (F1, F2, F3), ATP synthase subunit beta (G1), phosphoglycerate kinase, chloroplastic (G2, G3 and G4) and Oxygen-evolving enhancer protein 2 (G5).

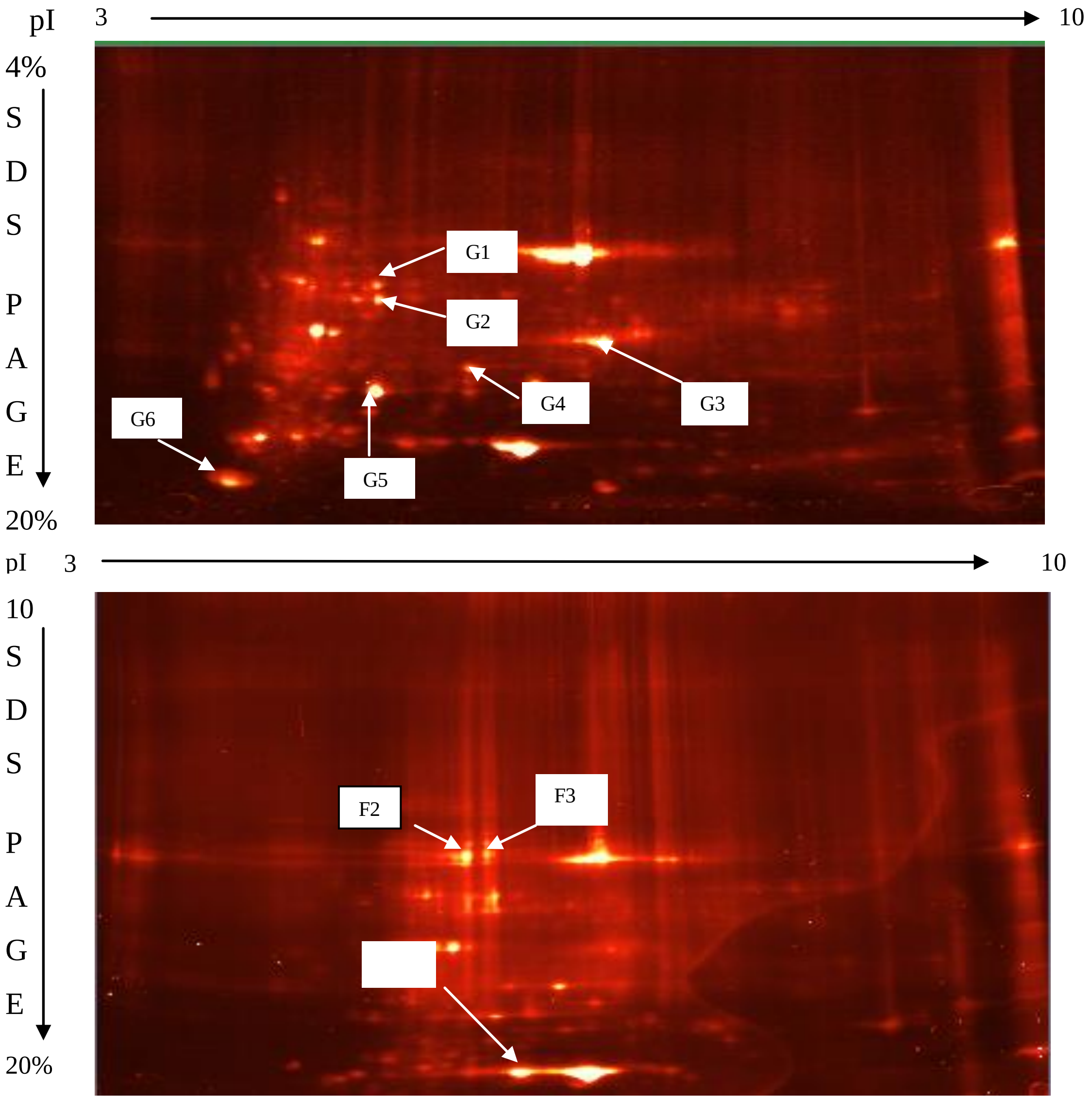


Figure 3.4: (A) 2-D gel of SST 347, Day 7 after leaf rust infection, G1-G6, up-regulated protein spots when compared to SST 356 and F1-F3 (B) up-regulated protein spots in SST 356 when compared to SST 347.

Table 3.2: Proteins identified from the wheat leaf proteome (SST 356) on day 7 after leaf rust infection

N	**Protein (s) identified**	**Accession number**	**% coverage (95%)**	**pI/Molecular[1] weight (Dalton)**	**Function[2]**	**References**
1	Cysteine synthase	P38076	3.691999987[b]	5.46/34114.38	Catalytic activity: O-acetyl-L-serine + hydrogen sulfide = L-cysteine + acetate.	Youssefian *et al*., 1993
2	Cytochrome b6-f complex iron-sulfur subunit, chloroplastic	Q7X9A6	11.71000004[a]	8.47/23726.26	Component of the cytochrome b6-f complex, which mediates electron transfer between photosystem II (PSII) and photosystem I (PSI), cyclic electron flow around PSI, and state transitions	Hird *et al*., 1991 Ogihara *et al*., 2000 Lu *et al*., 2002
3	Cytochrome f	P05151	14.37000036[b]	8.83/35362.89		
4	Histone H3.2	P68428	5.14700003*	11.29/15283.90	Core component of nucleosome. Nucleosomes wrap and compact DNA into chromatin, limiting DNA accessibility to the cellular machineries which require DNA as a template. Histones thereby play a central role in transcription regulation, DNA repair, DNA replication and chromosomal stability. DNA accessibility is regulated via a complex set of post-translational modifications of histones, also called histone code, and nucleosome remodeling.	Tabata *et al*., 1984 Modro *et al*., 1988
5	Elongation factor 1-beta	P29546	4.166999832[b]	4.54/23088.03	EF-1-beta and EF-1-beta' stimulate the exchange of GDP bound to EF-1-alpha to GTP.	Oizumi *et al*., 1992
6	2-Cys peroxiredoxin BAS1, chloroplastic (Fragment)	P80602	6.667000055*	5.71/23326.68	May be an antioxidant enzyme particularly in the developing shoot and photosynthesizing leaf	Tsunoyama and Toyoshima, 1997 Tsunoyama *et al*.,

						1996
7	Oxygen-evolving enhancer protein 2, chloroplastic	Q00434	3.875999898[a]	8.84/27269.68	May be involved in the regulation of photosystem II.	James and Robinson, 1991
8	Hypersensitive induced reaction protein 1	H9CDF7	4.301000014[b]	5.57/30735.17	HIR genes are known to be associated with the hypersensitive response and disease defence	Liu *et al*., 2011 Duan *et al*., 2013
9	Hypersensitive induced reaction protein 1	H9CDF6	4.224999994[b]	5.42/31325.80		
10	Photosystem II protein D1	P12463	16.15000069[b]	5.21/38920.62	Photosystem II (PSII) is a light-driven water: plastoquinone oxidoreductase that uses light energy to abstract electrons from H(2)O, generating O(2) and a proton gradient subsequently used for ATP formation. It consists of a core antenna complex that captures photons, and an electron transfer chain that converts photonic excitation into a charge separation. The D1/D2 (PsbA/PsbA) reaction center heterodimer binds P680, the primary electron donor of PSII as well as several subsequent electron acceptors.	Hanley-Bowdoin & Chua, 1998 Ogihara *et al*., 2000
11	Photosystem II D2 protein	Q36814	9.064999968[b]	5.34/39470.38		
12	Phosphoglycerate kinase, chloroplastic	P12782	10.20999998* 26.87999904[b]	6.57/49839.53	Catalytic activity: ATP + 3-phospho-D-glycerate = ADP + 3-phospho-D-glyceroyl phosphate.	Longstaff *et al*., 1989 Jones *et al*., 1995
13	ATP synthase epsilon chain, chloroplastic	P69443	72.99000025* 85.39999723[a]	5.20/15217.63	Produces ATP from ADP in the presence of a proton gradient across the membrane.	Howe *et al*., 1985 Ogihara *et al.*, 2000

14	ATP synthase subunit beta, chloroplastic	P20858	44.38000023[a]	5.06/53857048		
15	ATP synthase subunit alpha, chloroplastic	P12112	4.563000053* 13.28999996[b]	6.11/55295.52		
16	Ribulose bisphosphate carboxylase small chain clone 512 (Fragment)	P07398	89.38000202* 99.11999702[a] 52.20999718[b]	5.84/13055.05	RuBisCO catalyzes two reactions: the carboxylation of D-ribulose 1, 5-bisphosphate, the primary event in carbon dioxide fixation, as well as the oxidative fragmentation of the pentose substrate. Both reactions occur simultaneously and in competition at the same active site (By similarity).	Broglie *et al*., 1983 Smith *et al*., 1983 Terachi *et al*., 1987 Houtz *et al*., 1989 Mehta *et al*., 1992 Ogihara *et al*., 1991; 2000 Niu, 2003
17	Ribulose bisphosphate carboxylase small chain PW9, chloroplastic	P26667	60.5700016* 73.71000051[a] 39.43000138[b]	8.52/19454.45		
18	Ribulose bisphosphate carboxylase small chain PWS4.3, chloroplastic	P00871	50.56999922* 70.10999918[a] 33.91000032[b]	8.99/19417.36		
19	Ribulose bisphosphate carboxylase large chain	P11383	14.25999999* 10.68999991[a] 28.5100013[b]	6.22/52851.13		
20	Sedoheptulose-1,7-bisphosphatase, chloroplastic	P46285	18.07000041[b]	6.04/42060.82	Catalytic activity: Sedoheptulose 1,7-bisphosphate + H_2O = sedoheptulose 7-phosphate + phosphate.	Raines *et al*., 1992 Miles *et al*., 1993
21	Phosphoribulokinase, chloroplastic	P26302	3.465000167[b]	5.72/45141.39	Catalytic activity: ATP + D-ribulose 5-phosphate = ADP + D-ribulose 1, 5-bisphosphate.	Raines *et al*., 1989 Lloyd *et al*., 1991
22	ADP,ATP carrier protein 2, mitochondrial	Q41630	2.718999982[b]	9.78/35790.30	Transmembrane transport. Catalyses the exchange of ADP and ATP across the mitochondrial inner	Iacobazzi *et al*., 1996

23	ADP,ATP carrier protein 1, mitochondrial	Q41629	2.718999982[b]	9.80/35961.63	membrane	
24	Oxygen-evolving enhancer protein 1, chloroplastic 1	P27665	24.61999953* 5.84600009[a] 49.84999895[b]	8.73/34740.37	Stabilizes the manganese cluster which is the primary site of water splitting.	Meadows *et al.*, 1991

[*] % coverage of proteins in spot F1; [a] % coverage of proteins in spot F2; [b] % coverage of proteins in spot F3. 1&2 (pI/Molecular weight and function) were obtained from www.ExPASy.org.

Table 3.3: Proteins identified from the wheat leaf proteome (SST 347) on Day 7 after leaf rust infection

N	Protein (s) identified	Accession number	% coverage (95%)	pI /Molecular weight (Dalton)[1]	Function[2]	References
1	Cytochrome b6-f complex iron-sulfur subunit, chloroplastic	Q7X9A6	13.51000071[d]	8.47/23726.26	Component of the cytochrome b6-f complex, which mediates electron transfer between photosystem II (PSII) and photosystem I (PSI), cyclic electron flow around PSI, and state transitions.	Lu *et al*., 2002
2	Histone H2B.4	Q43215	6.667000055[d]	10.8/14816.46	Core component of nucleosome. Nucleosomes wrap and compact DNA into chromatin, limiting DNA accessibility to the cellular machineries which require DNA as a template. Histones thereby play a central role in transcription regulation, DNA repair, DNA replication and chromosomal stability. DNA accessibility is regulated via a complex set of post-translational modifications of histones, also called histone code, and nucleosome remodeling.	Brandt *et al*., 1988 Green *et al*., 1990 Yang *et al*., 1995
3	Histone H2B.5	Q43216	6.617999822[*,d,e]	10.03/14968.62		
4	Histone H2B.3	Q43217	6.521999836[d]	10.08/15104.73		
5	Histone H2B.2	P05621	5.999999866[d]	10.19/16226.50		
6	Histone H2B.1	P27807	5.92099987[d]	10.00/16433.26		
7	Flavone O-methyltransferase 1	Q84N28	28.33000124* 4.721999913[c]	5.75/38859.95	Flavone-specific O-methyltransferase with a preference for flavones > flavonols. Active with tricetin, luteolin, quercitin and eriodictyol. Very low activity with phenylpropanoids (5-hydroxyferulic acid and caffeic acid). Catalyzes	Jang *et al*., 2005 Zhou *et al*., 2009

					the sequential O-methylation of tricetin via 3'-O-methyltricetin, 3',5'-O-methyltricetin to 3',4',5'-O-trimethyltricetin	
8	Phosphoglycerate kinase, chloroplastic	P12782	52.07999945* 54.17000055[a] 49.16999936[c] 62.91999817[b] 292000204[d] 7,500000298[e]	6.57/49839.53	Catalytic activity: ATP + 3-phospho-D-glycerate = ADP + 3-phospho-D-glyceroyl phosphate.	Longstaff *et al*., 1989 Jones *et al*., 1995
9	S-adenosylmethionine synthase	B0LXM0	14.3900007[a] 16.41000062[b]	5.55/43179.90	Catalyzes the formation of S-adenosylmethionine from methionine and ATP. The overall synthetic reaction is composed of two sequential steps, AdoMet formation and the subsequent tripolyphosphate hydrolysis which occurs prior to release of AdoMet from the enzyme (By similarity).	Wang *et al*., 2008
10	Oxygen-evolving enhancer protein 2, chloroplastic	Q00434	14.7300005* 37.59999871[a] 23.63999933[b] 14.33999985[c] 64.34000134[d] 22.08999991[e]	8.84/27269.68	May be involved in the regulation of photosystem II.	James and Robinson, 1991
11	ATP synthase subunit	P12862	20.82999945*	5.70/55264.20	Mitochondrial membrane ATP synthase (F1F0	Schulte *et al*., 1989

	alpha, mitochondrial		3.536000103[a] 14.33999985[c] 18.46999973[d] 37.33000159[e]		ATP synthase or Complex V) produces ATP from ADP in the presence of a proton gradient across the membrane which is generated by electron transport complexes of the respiratory chain. F-type ATPases consist of two structural domains, F1 - containing the extramembraneous catalytic core, and F0 - containing the membrane proton channel, linked together by a central stalk and a peripheral stalk. During catalysis, ATP synthesis in the catalytic domain of F1 is coupled via a rotary mechanism of the central stalk subunits to proton translocation. Subunits alpha and beta form the catalytic core in F1. Rotation of the central stalk against the surrounding alpha3beta3 subunits leads to hydrolysis of ATP in three separate catalytic sites on the beta subunits. Subunit alpha does not bear the catalytic high-affinity ATP-binding sites (By similarity)	
12	Phosphoglycerate kinase, cytosolic	P12783	12.47000024* 24.1899997[a] 6,733000278[e]	5.64/42121.96	Catalytic activity: ATP + 3-phospho-D-glycerate = ADP + 3-phospho-D-glyceroyl phosphate.	Longstaff *et al*., 1989

13	Photosystem II CP43 reaction center protein	Q9XPS4	8.878999949* 6.341999769[a] 9.724999964[b] 3,382999822[e]	6.93/52001.04	One of the components of the core complex of photosystem II (PSII). It binds chlorophyll and helps catalyze the primary light-induced photochemical processes of PSII. PSII is a light-driven water:plastoquinone oxidoreductase, using light energy to abstract electrons from H2O, generating O2 and a proton gradient subsequently used for ATP formation	Matsuoka *et al*., 1999 Ogihara *et al*., 2000
14	Photosystem II D2 protein	Q36814	2.833000012[a] 5.38199991[c]	5.34/39470.38	Photosystem II (PSII) is a light-driven water: plastoquinone oxidoreductase that uses light energy to abstract electrons from H(2)O, generating O(2) and a proton gradient subsequently used for ATP formation. It consists of a core antenna complex that captures photons, and an electron transfer chain that converts photonic excitation into a charge separation. The D1/D2 (PsbA/PsbA) reaction center heterodimer binds P680, the primary electron donor of PSII as well as several subsequent electron acceptors.	Hanley-Bowdoin & Chua, 1998 Ogihara *et al*., 2000
15	Photosystem II protein D1	P12463	5.38199991* 8.782000095[c]	5.12/38920.62		
16	Catalase	P55313	2.641999908[a]	6.58/56479.86	Occurs in almost all aerobically respiring organisms and serves to protect cells from the toxic effects of hydrogen peroxide	Song *et al*., 1996
17	Adenylosuccinate	O24396	2.101000026	5.9350917.86	Plays an important role in the de novo pathway	Fonne-Pfister *et al*.,

	synthetase, chloroplastic (Fragment)				and in the salvage pathway of purine nucleotide biosynthesis. Catalyzes the first committed step in the biosynthesis of AMP from IMP (By similarity).	1996 Prade *et al*., 2000
18	ATP synthase subunit beta, chloroplastic	P20858	73.08999896* 48.19000065[a] 62.25000024[b] 76.91000104[c] 9.1200012[d,e] 9.036000073[e]	5.06/53857048	Produces ATP from ADP in the presence of a proton gradient across the membrane. The catalytic sites are hosted primarily by the beta subunits.	Howe *et al*., 1991; Ogihara *et al*., 2000
19	ATP synthase subunit alpha, chloroplastic	P12112	33.93000066* 35.12000144[a] 18.6499998[c] 29.17000055[b] 15.08000046[d] 32.53999949[e]	6.11/55295.52		
20	ATP synthase epsilon chain, chloroplastic	P69443	14.59999979* 8.028999716[d]	5.20/15217.63		
21	Cysteine synthase	P38076	11.99999973* 9.538000077[d]	5.46/34114.38	Catalytic activity: O-acetyl-L-serine + hydrogen sulfide = L-cysteine + acetate.	Youssefian *et al*., 1993
22	Ribulose bisphosphate carboxylase small chain PW9, chloroplastic	P26667	41.71000123* 31.43000007[a] 14.85999972[b]	8.52/19454.45	RuBisCO catalyzes two reactions: the carboxylation of D-ribulose 1, 5-bisphosphate, the primary event in carbon dioxide fixation, as	Broglie *et al*., 1983 Smith *et al*., 1983 Terachi *et al*., 1987

			23.99999946^{d} 30.86000085^{e}		well as the oxidative fragmentation of the pentose substrate. Both reactions occur simultaneously and in competition at the same active site (By similarity).	Houtz *et al*., 1989 Mehta *et al*., 1992 Ogihara *et al*., 1991; 2000 Niu, 2003
23	Ribulose bisphosphate carboxylase small chain clone 512 (Fragment)	P07398	48.66999984* 32.73999989^{a} 14.15999979b 38.94000053^{c} 38.94000053^{e}	5.84/13055.05		
24	Ribulose bisphosphate carboxylase small chain PWS4.3	P00871	31.61000013* 21.25999928^{a} 9.194999933^{b} 25.29000044^{c} 18.38999987^{d} 25.29000044^{e}	8.99/19417.36		
25	Ribulose bisphosphate carboxylase large chain	P11383	17.61000007* 20.96000016^{a} 22.64000028^{b} 15.51000029^{c} 40.88000059^{d} 24.10999984^{e}	6.22/52851.13		
26	Sedoheptulose-1, 7-bisphosphatase, chloroplastic	P46285	21.87999934* 11.69999987^{b} 8.651000261^{c}	6.04/42060.82	Catalytic activity: Sedoheptulose 1,7-bisphosphate + H_2O = sedoheptulose 7-phosphate + phosphate.	Raines *et al*., 1992 Miles *et al*., 1993
27	Phosphoribulokinase,	P26302	12.3800002^{a}	5.72/45141.39	Catalytic activity: ATP + D-ribulose 5-phosphate	Raines *et al*., 1989

	chloroplastic		6.436000019[b]		= ADP + D-ribulose 1,5-bisphosphate.	Lloyd *et al*., 1991
28	Oxygen-evolving enhancer protein 1, chloroplastic	P27665	15.99999964* 5.538000166[a]	6.22/34740.37	Stabilizes the manganese cluster which is the primary site of water splitting.	Meadows *et al*., 1991

*% coverage of proteins spot G1, [a]% % coverage of proteins spot G2, [b]% coverage of proteins spot G3, [C]% coverage of proteins spot G4, [d]% coverage of proteins spot G5, [e]% coverage of proteins spot G6.1&2 (pI/Molecular weight and function) were obtained from www.ExPASy.org.

3.3.4 Summary of differentially expressed proteins by function in response to leaf rust infection

Differentially expressed proteins identified by MS in the resistant and susceptible cultivar in response to stress imposed by leaf rust infection were summaried according to their functional groups **(**Figure 3.5).

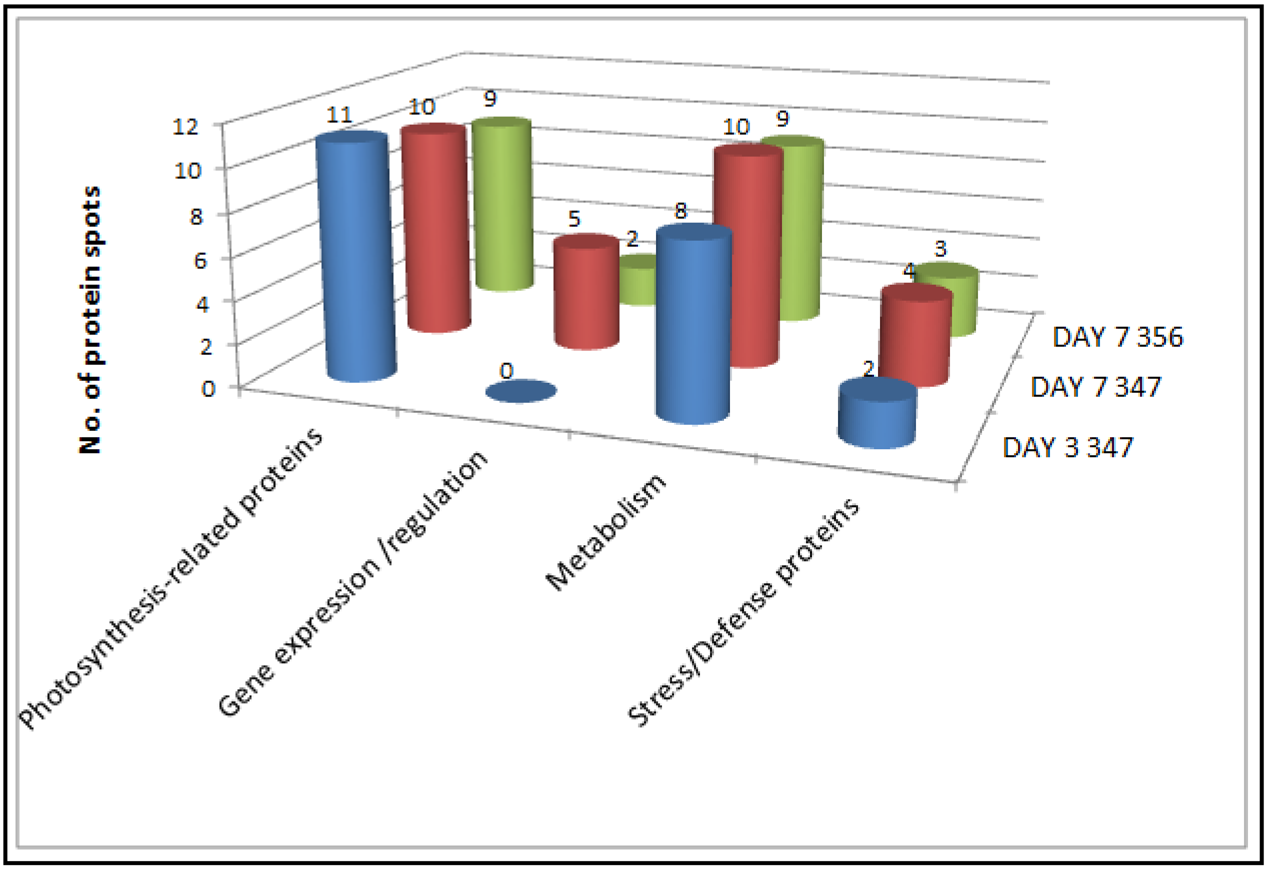


Figure 3.5: A graphical representation of differentially expressed proteins identified in both leaf rust infected resistant and susceptible wheat plants.

3.4 DISCUSSION

3.4.1 General stress-related defence responses

Most spots detected had keratin, trypsin and other low matching contaminants including *P. triticina* proteins that are not discussed further. Keratin, a fibrous structural protein abundant on the outer layer of the skin of humans (*Homo sapiens*), in hair and nails could have been introduced through sample preparation and this has often been seen as a source of contamination in MS (Hogde *et al*., 2013). The other common external protein contaminant, trypsin was used in the experimental procedures for the proteolytic digestionof the samples to digest proteins into peptides for analysis (Hogde *et al*., 2013). *P. triticina* were identified in most samples since the spores could not be removed before proceeding with the analysis. From the 12 spots analysed, a total of 39 proteins were identified. According to their functions, most of the proteins identified were photosynthesis-related including photosytem II reaction protein, cytochrome, oxygen evolving enhancer protein and ribulose bisphosphate carboxylase (Figure 3.5). Trumble *et al*. (1993) and Shen *et al*. (2003) reported inducible expression of photosynthetic genes following pathogen attacks. The up-regulation could be attributed to the fact that the chloroplast was affected by the pathogen and in order for the plant to compensate for the loss, it had to increase the rate photosynthesis. However, expression levels reduced from Day 3 through Day 7 for both the resistant and susceptible cultivars. This was not surprising as some authors have also reported a decrease in photosynthetic gene expression following attack by pathogens or insects (Zhou *et al*., 2006; Nabity *et al*., 2009; Bilgin *et al.*, 2010; Bazargani *et al*., 2011; Li *et al*., 2011). This type of defence has been termed “hidden cost” of defence by Bilgin *et al*. (2008) as the photosynthetic capacity is reduced to allocate resources from growth to defence. This reduction in photosynthesis related proteins could be due to the onset of HR which is well known as a rapid and effective defence mechanism by plants to limit colonisation by biotrophic fungi (Zou *et al*., 2005; Liu *et al*., 2011). Lisa *et al*. (1999) also reported a decrease in photosynthesis-related genes during HR.

Gene expression and regulation proteins were upregulated but only appeared at Day 7 post infection in both the resistant and susceptible cultivars, although 5 of the proteins were expressed in the resistant cultivar as opposed to 2 in the susceptible. A number of biochemical changes are known to contribute to early responses of the plant host following pathogen recognition with one of the first reaction being rearrangement of gene expression and regulation for transcriptional and posttranslational activation of defence (Zhu *et al*., 1996; Maytalman *et al*., 2013). Also identified were wheat histone proteins (H2B, #6) that help in the condensed packing of DNA and in transcription regulation, DNA replication and repair. In addition, histones are known to exhibit antimicrobial activities (Kawasaki and Iwamoto, 2008). De Lucca *et al*. (2011) in a study showed that wheat histones (H1-H4) had antifungal activity against *Fusarium graminearum*.

There was also an upregulation of proteins involved in the primary metabolic pathways including biosynthesis of carbohydrate and energy metabolism. Cysteine synthase and adenylosuccinate synthetase are enzymes involved in amino acid biosynthesis. ATP synthase is an important enzyme of energy metabolism which produces ATP from ADP in the presence of a proton gradient across membranes. Phosphoribulokinase and sedoheptulose-1,7-biophosphatase are two important proteins in the Calvin cycle, which are involved carbohydrate biosynthesis. Phosphoglycerate kinase is an important protein in the Kreb cycle and is involved in step 2 of the sub-pathway that synthesises pyruvate from D-glyceraldehyde-3-phosphate. Kinases regulate recognition and early responses to diverse signals, and frequently play key roles in various developmental and physiological processes including defence (Garcia *et al*., 2012). The increased expression of these proteins could be ascribed to the plant defending itself and preventing the pathogen from spreading. Zhu *et al*. (2015) found out that there was a positive contribution of the kinase TaAGC1 to wheat immunity to the necrotrophic fungus *Rhizoctonia cerealis*.

Additional six wheat proteins, 2-cys peroxiredoxin BAS1, sedoheptulose-1, 7-bisphasphatase, S-adenosylmethionine synthase, Flavone O-methyltransferase, Hypersensitive induced reaction protein 1, catalase-1 and catalase that are defence and oxidative stress proteins were also identified. Four of these proteins (S-adenosylmethionine synthase, Flavone O-methyltransferase, catalase-1 and catalase) were identified only in the resistant cultivar where as in the susceptible two hypersensitive induced reaction protein, 2-cys peroxiredoxin BAS1 and sedoheptulose-1, 7-bisphosphatase were identified. An implication of this is that, the latter proteins are involved in general wheat defence and not involved in the resistance mechanism of wheat to stress imposed by leaf rust infection.

2-cys peroxiredoxin (2-CP) constitutes a family of enzymes known to catalyse the transfer of electrons from sulfhydryl residues to peroxides and are ubiquitously distributed in all organisms (Baier and Diet, 1996). They are a group of enzymes with catalytic function in the detoxification of cell toxic peroxides and have been identified as members of the antioxide defence of the chloroplast (Dietz *et al.,* 2002). Peroxiredoxins play a key role in combating ROS generated at the level of electron transport in plants exposed to diverse types of biotic and abiotic stresses (Bazargani *et al*., 2011). Maytalman *et al*. (2013) demonstrated that in an incompatible interaction, the overpression of Prx Q and type II Prx were observed to maintain low peroxide concentrations outside the sites of infection and spare the uninfected cells.

3.4.2 Pathways identified in wheat in response to leaf rust infection

S-adenosylmethionnine synthase, ATP synthase, phosphogycerate kinase, phosphoribulokinase, sedoheptulose -1, 7, bisphosphatase, 2-cys peroxiredoxin BAS1 and cysteine synthase pathways that were activated in wheat in response to stress imposed by RWA feeding were also activated following leaf rust infection. Other proteins that were activated, that were unique to pathogen attack, include adenylosuccinate and catalase. These are involved in various metabolic pathways including purine biosynthesis and reactive oxygen species degradation.

3.4.2.1Purine biosynthesis

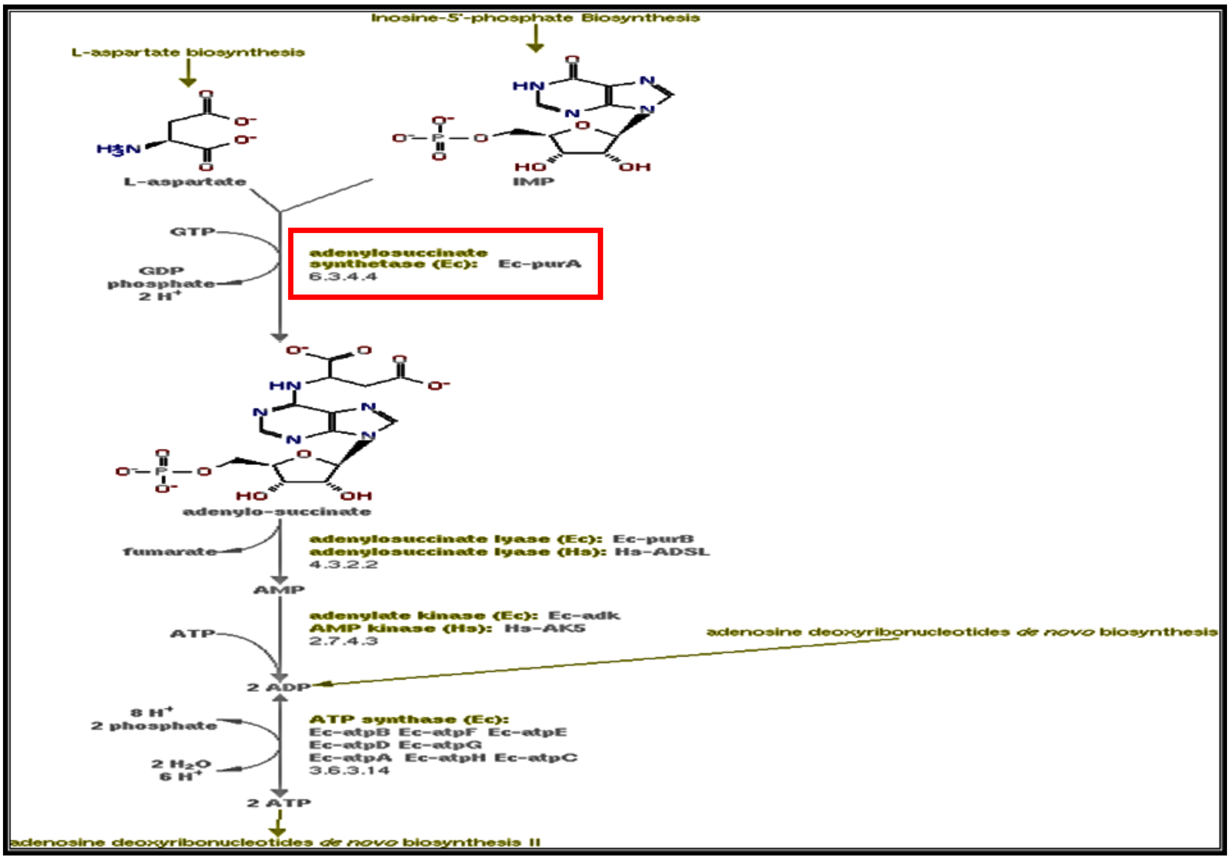


Figure 3.6: Enzymes up-regulated in purine biosynthesis pathway (MetaCyc: www.metacyc.org; Caspi *et al.*, 2014) in response to leaf rust infection; adenylosuccinate synthetase reaction: *L-aspartate* + *IMP* + *GTP* → *adenylo-succinate* + *GDP* + *phosphate* + *2* H^+.

Adenylosuccinate synthase is an enzyme that plays an important role in purine bisoynthesis. It catalyses the guanosine triphosphate (GTP)-dependent conversion of inosine monophosphate (IMP) and aspartic acid to guanosine phosphate (GPD), phosphate and N (6)-(1, 2-dicarboxyethyl)-AMP. IPM is the first purine nucleotide that is synthesised *de novo* that is converted to adenylosuccinate by the enzyme adenylosuccinate synthase that is later converted to the first adenosine nucleotide (AMP) by the action of adenylosuccinate lyase (Prade *et al.*, 2000). AMP is phosphorylated to ADP then to ATP with the first reaction is catalysed by adeneyate kinase. Reactions that convert ADP to ATP are numerous and are found in multiple pathways but the major source for this conversion is the ATP synthase pathway.

3.4.2.2 Reactive oxygen species (ROS) degradation

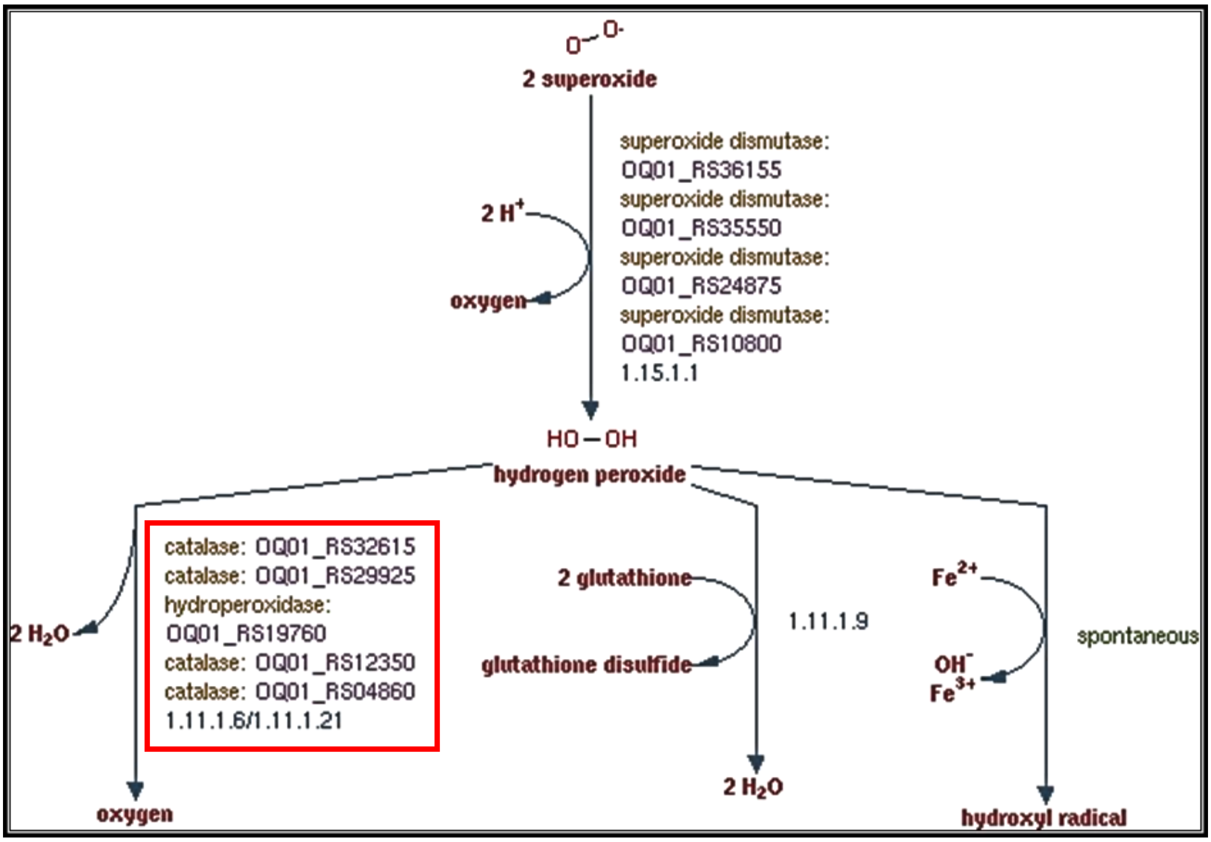


Figure 3.7: Enzyme up-regulated in ROS degradation pathway (MetaCyc: www.metacyc.org; Caspi *et al.*, 2014) in response to leaf rust infection. Catalase reaction: $2H_2O_2 \rightarrow 2H_2O + O_2$

ROS are unstable and extremely reactive with the chemical reactivity inducing lipid peroxidation and protein oxidation/degradation. Superoxide arising from the mitochondria respiratory chain constitutively present in cells; hydrogen peroxide arising from dismutation of oxygen or the action of oxidase enzymes and the highly reactive hydroxyl radical from the decomposition of hydrogen peroxides are the three main types of ROS. Hydrogen peroxide (H_2O_2) is an important signalling molecule involved in plant development and environmental responses (Mhamdi *et al.*, 2010). ROS activates programmed cell death (PCD) associated with normal development and immune responses that clear pathogen and destroys infected cells. At high concentrations, H_2O_2 is toxic to the plant and plants therefore secrete catalase which cleaves H_2O_2 to water and oxygen (Chelikani *et al.*, 2004). Catalase is therefore is therefore and important enzyme that protects the cell from oxidative damage by ROS. Nishikawa *et al.* (2009) in a study conducted on salt-stressed wheat chloroplast observed an increase in the levels of five subunits of catalase, a well-characterised antioxidant enzyme that proteceted the cells from toxic effect of H_2O_2. The up regulation of catalase is evident that the ROS/NO pathway has been activated see section1.6.1, Figure1.6.

2.5 Conclusion

Our results showed that stress response proteins are significantly up-regulated in resistant diploid wheat (*Triticum aestivum)* cultivar SST 347 in response to attack by leaf rust. Garcia-Burgger *et al*. (2006) and Thomma *et al.* (2009) showed that pathogen infection leads to phosphorylation, membrane depolarization, calcium influx and release of reactive oxygen species (ROS) resulting in the activation of phytohormone-dependent pathways incuding ET, JA and SA. Pathways linked to oxygen reactive species (ROS) were identified indicating that one of the early responses of wheat to leaf rust infection is the induction of hypersensitive response. Most of the proteins identified in wheat response to leaf rust infection were mostly associated with the chloroplast. In future, the chloroplast proteome will be studied.

CHAPTER FOUR

LEAF RUST AND RUSSIAN WHEAT APHID POPULATION

4.1 Introduction

Plants and insects are intimately associated in the natural ecosystems and interact in a complex way. In this interaction, some insects are beneficial to plants in that they defend the plant and assist in pollination while plants in turn provide food and shelter, oviposition sites for the insects (Panda and Khush, 1995). However, plants may be severely damaged in the increase intensity of attacks, as such for millions of years, plants have therefore been battling with these invaders thereby developing various defence mechanisms (Boyd, 2012; War *et al*., 2012). Like animals, they develop a defence system capable of recognising non-self-molecules or signals from damaged cells and activate plant immune response against the herbivore (Hare, 2011; Howe and Jander, 2008; Verhage and van Wess, 2010).

In order for plants to protect themselves from damage, they have developed a wide variety of direct and indirect defences. Plants direct defences are mediated by features that affects the herbivore biology such as mechanical protection on plant surfaces such as hairs, trichomes, thorns, spines and thicker leaves or by the production of toxic chemicals including terpenoids, alkaloids, anthocynanins, phenols and quinones that either kill or retard the development of the herbivores (Hanley *et al*., 2007). On the other hand, indirect defences against insects are mediated by the release of a mixture of volatiles that specifically attract natural enemies of the herbivore and/or by providing food such as extra floral nectar and housing to augment the efficiency of the natural enemies (Arimira *et al.,* 2009).

Plants respond to herbivore attack through an intricate and dynamic defence system that include structural barriers, toxic chemicals and attraction of natural enemies of the target pests (Hanley *et al*., 2007; Howe and Janer, 2008; Karban, 2011). Both defence mechanisms (direct or indirect) may be present constitutively or induced after damage by the herbivores. Constitutive (always present) defences include many preformed barriers such as cell walls and waxy epidermal cuticles acting as first line defences repelling herbivores through direct toxicity or by reducing the digestibility of plant tissue (Usha and Jyothsna, 2010; War *et al*., 2011). In addition to these preformed barriers virtually all living plant cells are endowed with the ability to detect invading pathogens and respond with inducible defences including the production of toxic chemicals, pathogen-degrading enzymes and deliberate cell suicide (Hanley *et al*., 2007; Freeman and Beattie, 2008). However, plants often wait until an attacker is detected before producing toxic chemicals or defence-related proteins because of the high energy costs and nutritional requirements associated with their production and maintenance (Freeman and Beattie, 2008).

The constitutive defences constitute basal resistance otherwise known as innate immunity and can be triggered when plant cells recognised microbe-associated molecular patterns (MAMPs) including specific proteins such as lipopolysaccharides, and cell wall components commonly found in microbes resulting in fortification of plant cells against attack. However, non-pathogens as well as pathogens can trigger basal resistance in plants due to the widespread presence of the molecular components in their cells. Secondary metabolites are compounds that do not affect the normal growth and development of a plant but are known to perform useful functions for the plant and can be either constitutively stored as inactive forms or induced in response to the insect or microbe attack (Howe and Jander, 2008). Secondary plant compounds act as insect repellents, feeding inhibitors and/or toxins. Plant toxic compounds have been classified into chemically-derived substances and protein-derived molecules. Indirect plant defence mechanisms against herbivore rely on the release of volatile organic compounds (VOCs) (Shiojiri *et al*., 2012) in order to signal the presence of herbivores to their predators and parasitoids. These insects might feed on or deposit eggs into the larvae of the herbivorous insect (Birkett *et al*., 2000; Dangl and Jones, 2001).

Pathogens have developed counter measures that are able to suppress basal resistance in many plant species and as such the plant may respond with another line of defence; the hypersensitive response (HR) characterised by deliberate plant cell suicide at the site of infection. Although HR is drastic when compared to basal resistance, it limits pathogen access to water and nutrients by foregoing a few cells in order to save the plant. HR typically is more pathogen specific than the basal defence and is often triggered when gene products in the plant cell recognize the presence of a specific disease-causing effector molecule introduced into the host by the pathogen. A key factor of HR is that once it has been triggered, the plant tissue may become highly resistant to a broad range of pathogens for an extended period and this is known as systemic acquired resistance (SAR) and represents a heightened state of readiness in which plant resources are mobilised in case of further attack (Freeman and Beattie, 2008).

Wheat (*Triticum aestivum* L. and *T. durum*) is one of the main sources of calories and protein worldwide and is the second most important cereal cultivated throughout the Republic of South Africa (Department of Agriculture, Forestry and Fisheries, 2013; Chaves *et al*., 2013). However, production is severely constrained by both abiotic and biotic stresses. Of the biotic stresses, a devastating pest, Russian wheat aphid (RWA) and rust diseases are currently the major problem for wheat producers in the country. Severe infestations by RWA may result in yield losses of up to 90% on susceptible cultivars while *Puccinia triticina* infected fields may suffer 70% crop loss (Panvar *et al*., 2013). These two factors combined seriously affect wheat farmers' income as a result of the heavy reliance on pesticides that increase the production cost. Insect pest and wheat rust diseases are therefore a real threat to food security. Consequently, knowledge of pests and diseases that may cause injuries and likely affect plant health and quality is critical in minimising the gap between attainable yield and actual yield (Duveiller *et al*., 2007).

Leaf rust is able to cause extensive yield losses, both in quantity and quality when environmental conditions are favourable for its development (ARC-SGI, 2015). The disease is generally more severe on autumn-grown wheat in the winter rainfall regions of the Cape Province, but moist conditions and elevated temperatures during spring can promote leaf rust in the Free State. Irrigated spring wheat throughout South Africa can also be subjected to severe leaf rust epidemics under favourable conditions. The development and release of leaf rust resistant cultivars in South Africa has greatly minimised the risk posed by this disease to producers (ARC-SGI, 2015). Wheat varieties produced could either be Resistant (R), Moderately Resistant/Susceptible (MR or MS) or completely susceptible (S) to infection. A resistant variety retards or kills the fungus and the infection is stopped. These varieties may develop yellowish-white flecks at the site of spore penetration while a moderately resistant variety develops yellowish-white spots with a small amount of spore produced in the center. Moderately susceptible varieties develop small reddish-orange pustules surrounded by a yellow-white halo. Susceptible varieties on the other hand do not have the ability to retard fungal growth, as such the fungus grows extensively and produces relatively large pustules that may produce approximately 1,000 spores daily with each capable of re-infecting wheat.

As a result of this, together with favourable weather conditions for rust development, the disease increases rapidly and epidemics may occur. *Puccinia triticina* populations are genetically diverse with different races having the ability to cause infection on varieties with different resistance genes (Kolmer, 2013). Because of the dynamic nature of the leaf rust pathogen (races constantly changing), varieties formerly considered resistant can become susceptible if new rust races develop.

Russian wheat aphid (*Diuraphis noxia*) is one of the most important wheat pest globally distributed. Since its first report in South Africa in 1978, to date three other biotypes have emerged (Du Toit and Walters, 1984; Tolmay *et al*., 2007; Jankielsohn, 2011, 2014). The emergence of new biotypes is a challenge to the South African wheat industry as resistance in wheat that offered wheat producers a long-term solution to RWA control, might no longer be effective in areas where the new biotypes occur (Jankielsohn, 2011). This necessitates a critical determination of the diversity and extent of RWA biotype distribution in South Africa in order to successfully manage the pest by planting resistant cultivars. Several strategies are employed for the control of RWA infestation including cultural practices, chemical control, biological control as well as the use of resistant cultivars (Peairs *et al*., 2006; Hajek *e al*., 2007; Botha *et al*., 2014; Puterka *et al*., 2015). The RWA feeds within rolled leaves, thus making the use of chemicals for control a challenge. In South Africa, damage to wheat crops could be limited by the use of systemic insecticides (Quinn *et al*., 2011). However, the large-scale use of insecticides has since been discontinued as farmers both in commercial and small-scale production now plant resistant cultivars to control the pest. However, the development of new resistant cultivars does not keep abreast with the development of new breaking biotypes. Therefore, to avoid this problem it is possible to prime the plant defences before pest attack.

Induced resistance could be exploited as an important tool for pest management to minimize the increase use of insecticides for pest control (Sharma, 2009; Agrawal, 2011; War *et al*., 2012). This could be done by developing wheat cultivars which readily produce the inducible response upon mild infestation. This would form part of an integrated pest management for sustainable crop production. Researchers have learned to artificially trigger SAR by spraying plants with chemicals called activators or elicitors of induced resistance to build up the natural defence system against damage caused by herbivores (War *et al*., 2012). These compounds are gaining ground in the agricultural sector since they are less toxic to the environment than fungicides, insecticides and their protective effect can last much longer. Although chemicals have been used to induce SAR in plants, the use of biological agents has not been exploited. This study therefore attempts to find out whether prior infection of wheat with leaf rust has an influence on aphid infestation under laboratory conditions.

4.2 Materials and Methods

4.2.1 Experimental design

The experiment was conducted in a controlled environment (Conviron S10H, Controlled Environments Limited, Winnipeg, Manitoba, Canada) at the Department of Biochemistry and Microbiology, University of Fort Hare. Two wheat cultivars, SST 347 (resistant to Russian wheat aphid biotype SA1 and Leaf rust race 3SA145) and SST 356 (Moderately resistant to Russian wheat aphid biotype SA1 and susceptible to Leaf rust race 3SA145) were used in this study with seeds supplied by SENSAKO, Bethlehem. The rust used in this study was leaf rust (*P. triticina* pathotype 3SA145); the spores and the susceptible wheat line Morocco were obtained from the Agricultural Research Council-Small Grain Institute, Bethlehem, South Africa.

4.2.2 Cultivation of plant and leaf rust inoculation

Twelve seeds of the two cultivars were germinated in each of four pots containing sterile hygromix soil with 3 plants per pot. The plants were grown in a growth chamber (Conviron S1OH) set at 22 °C day/night, light intensity of 352 µMOL, 60% humidity and ambient $CO_{2.}$ Nutrients were supplied by watering the plants twice a week with hygromix (1g/L). Wheat seedlings were inoculated 14 days after germination with urediniospores of *P. triticina* race 3SA145 suspended in Soltrol-170® at the ARC-SGI using the method described by Terefe *et al*. (2014). The urediniospores suspension of the fungus was sprayed onto all seedlings in a pot (not onto individual seedlings or individual leaves) using a spray pump in an inoculation chamber until run off to ensure all seedlings were inoculated. The inoculated plants were first dried-off for 2 hrs under light for the moisture to evaporate and then incubated overnight in a dew chamber at ±20 °C and 100 % relative humidity. The infected plants were removed from the dew chamber dried for 3 hrs and placed in cubicles in a Conviron at constant temperature (22 °C).

4.2.3 Infestation of infected plants with RWA SA1

Infected susceptible and resistant wheat plants with leaf rust race 3SA145 were infested with RWA SA1 3, 5, 7 and 9 days post infections. Ten aphids were introduced to each experimental plant by cutting sections of leaves from a growing colony with ten aphids and placing them onto the new host plant. All three plants of each treatment were in the same pot. The control plants for this experiment were un-inoculated plants that were infested the same way as the tested plants. Both experimental and control plants were placed in insect cages and allowed to grow in the conviron under controlled environmental conditions and aphids were then allowed to feed and reproduce on the leaves up to 21 days. Twenty-four hours post infestation, population increase from the original (10 aphids) were determined per plant by manually counting the total number of aphids on the abaxial and adaxial surfaces of each leaf (per plant) carefully with the aid of a hand lens. This was continued once a day for the entire period of the study (21 days).

4.2.4 Assessment of disease severity

Visible manifestations of disease severity and aphid feeding damage were simultaneously assessed by examining chlorosis and leaf roll on each experimental plant under each treatment. Disease responses in seedlings inoculated with urediniospores of *P. triticina* were scored 14 days post infection using the 0 to 4 infection type scale (Terefe *et al*., 2009). Infection types of 0-2 would be considered low while infections types of 3-4 would be considered to be high.

4.3 RESULTS

4.3.1 Plant material

SST 347 is a wheat cultivar released in 2004 by Sensako with excellent yield potential, longer growth period and suited for early planting dates. To date, SST 347 is still resistant to RWA biotype SA1 and leaf rust race 3SA145. On the other hand, SST 356 was released in 2005 with a high yield potential, good disease resistance to leaf rust and moderately resistant to most of the economically important pests in the winter wheat growing areas, such as RWA SA1. However, it is currently susceptible to leaf rust race 3SA145 and only moderately resistant to RWA SA1 (Terefe *et al.*, 2014).

4.3.2 Host plant resistance

Wheat genotypes differed in their degree of resistance or susceptibility to infestation/infection. Infection types of the *P. triticina* race 3SA145 on the two wheat cultivars were recorded although the leaf rust resistance gene (s) in these cultivars is not known. Infection type of ;1+ and 3++ were observed for the wheat cultivar SST 347 and SST 356 respectively.

No visible symptoms were seen on both cultivars at twenty hours after the plants were inoculated (Figure 4.1). This was probably due to the fact that the infection was not yet established. From day 3 post infection symptoms started to appear on both cultivars. On the resistant cultivar, from day 3 upto day 9 post inoculation infection types 1-2 were observed confirming the fact that this cultivar is resistant to *P. triticina* race 3SA145. This infection type is characterised by hypersentive flecks that ranged from small to moderate size uredinia surrounded by chlorosis and necrosis (Figure 4.1). Large uredinia without surrounding chlorosis or necrosis (IT 3++) were observed on the leaf tissue of the moderately resistant cultivar, which is an indication of susceptibity (Figure 4.1). These physical symptoms support the proteomic data reported in the earlier chapter (chapter 3). The majority of the proteins found to be up regulated through out the experiment were photosynthesis-related proteins (Figure 3.5), which could indicate response from the plant to repair or minimise the onset of the chlorosis (loss of chlorophyll) observed. The observed 1+ scored for the resistant cultivar (SST 347) was also supported by the fact that more stress/defense related proteins were up regulated later during the experiment. These proteins elicited a hypersensitive response which was seen as small hypersensitive flecks on the resistant cultivar.

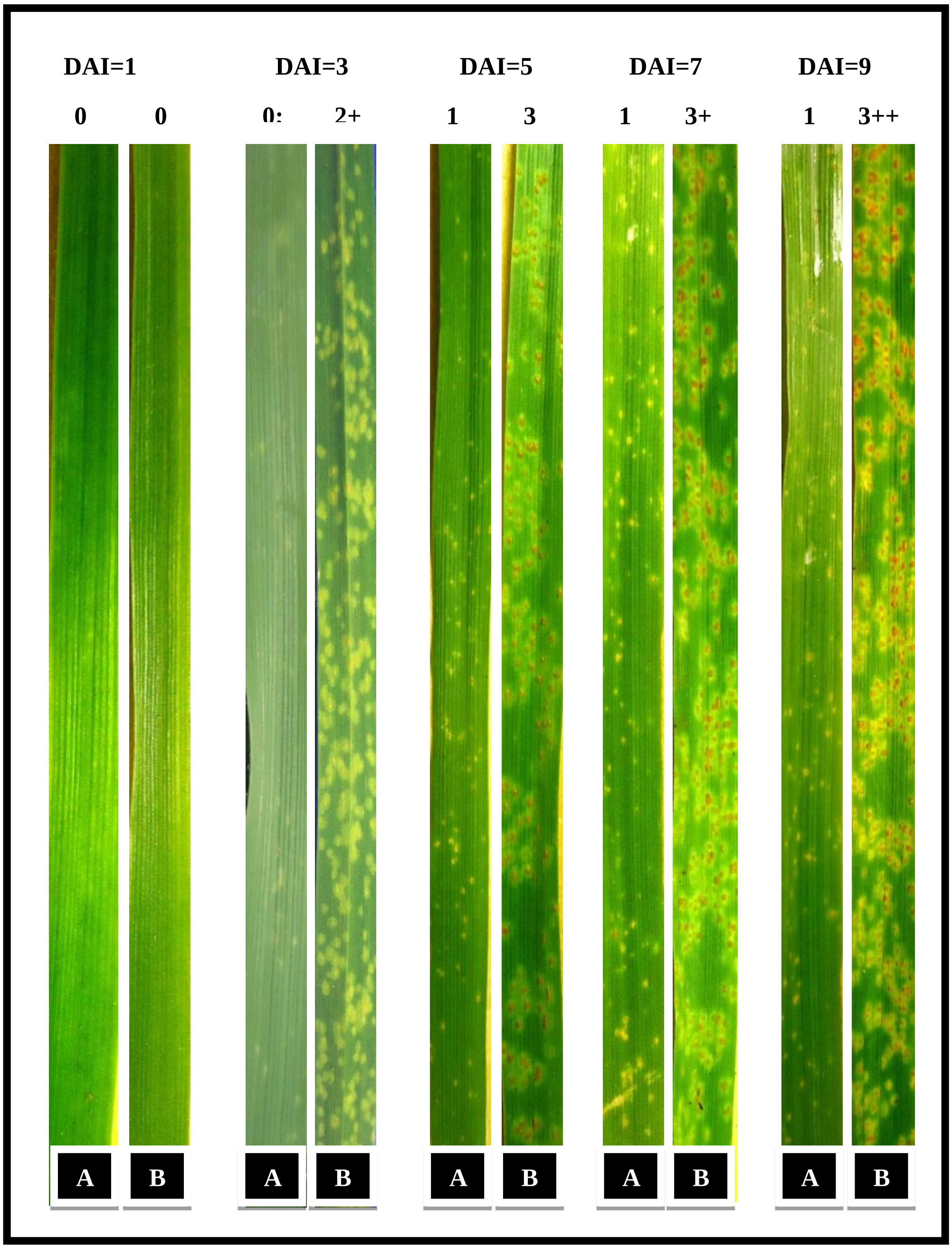


Figure 4.1: Damage rating on SST 347 (A) and SST 356 (B): 0-2, low infections; 3-4, high infections

4.3.3 Effects of Leaf rust (Lr) infection on Russian wheat aphid population growth on the resistant cultivar (SST 347)

Aphid population build up to the re-gain initial dose occurred at day three post infestation on the treatments with no prior Lr infection while for the treatments with prior Lr infection, the build-up was between 15-16 days after infection (DAI) (Figure 4.3).

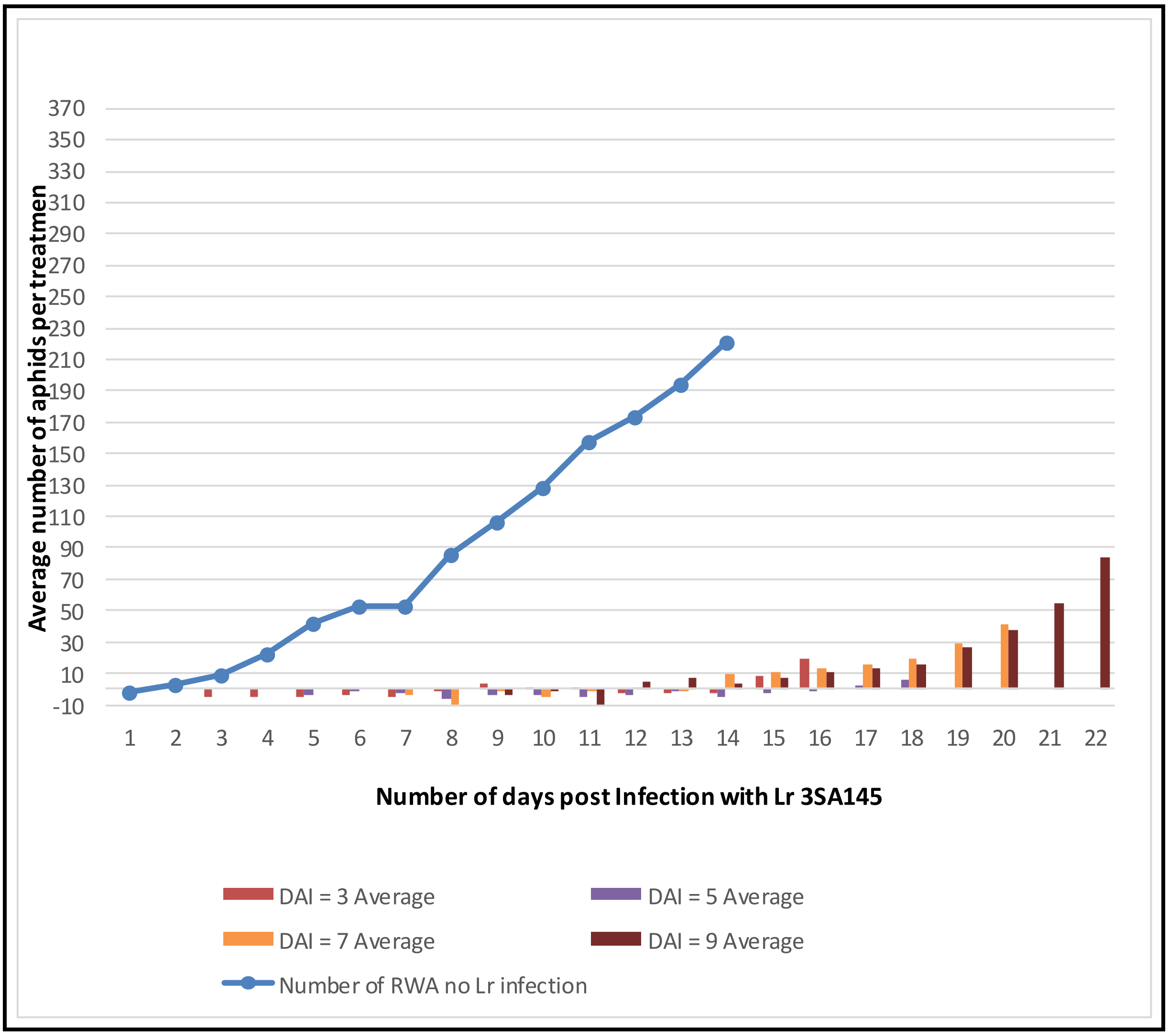


Figure 4.2: RWA SA1 population growth curves on SST 347 wheat cultivar with and without pre infection with *P. triticina* race 3SA145.

2.3.4 Effects of Leaf infection on Russian wheat aphid population growth on the moderately resistant aphid cultivar (SST 356)

Aphid population built up to the initial dose occurred at day one post infestation on the plants with no prior Lr infection while for the plants with prior infection with Lr, the build-up occurred between 12-13 DAI (Figure 4.4).

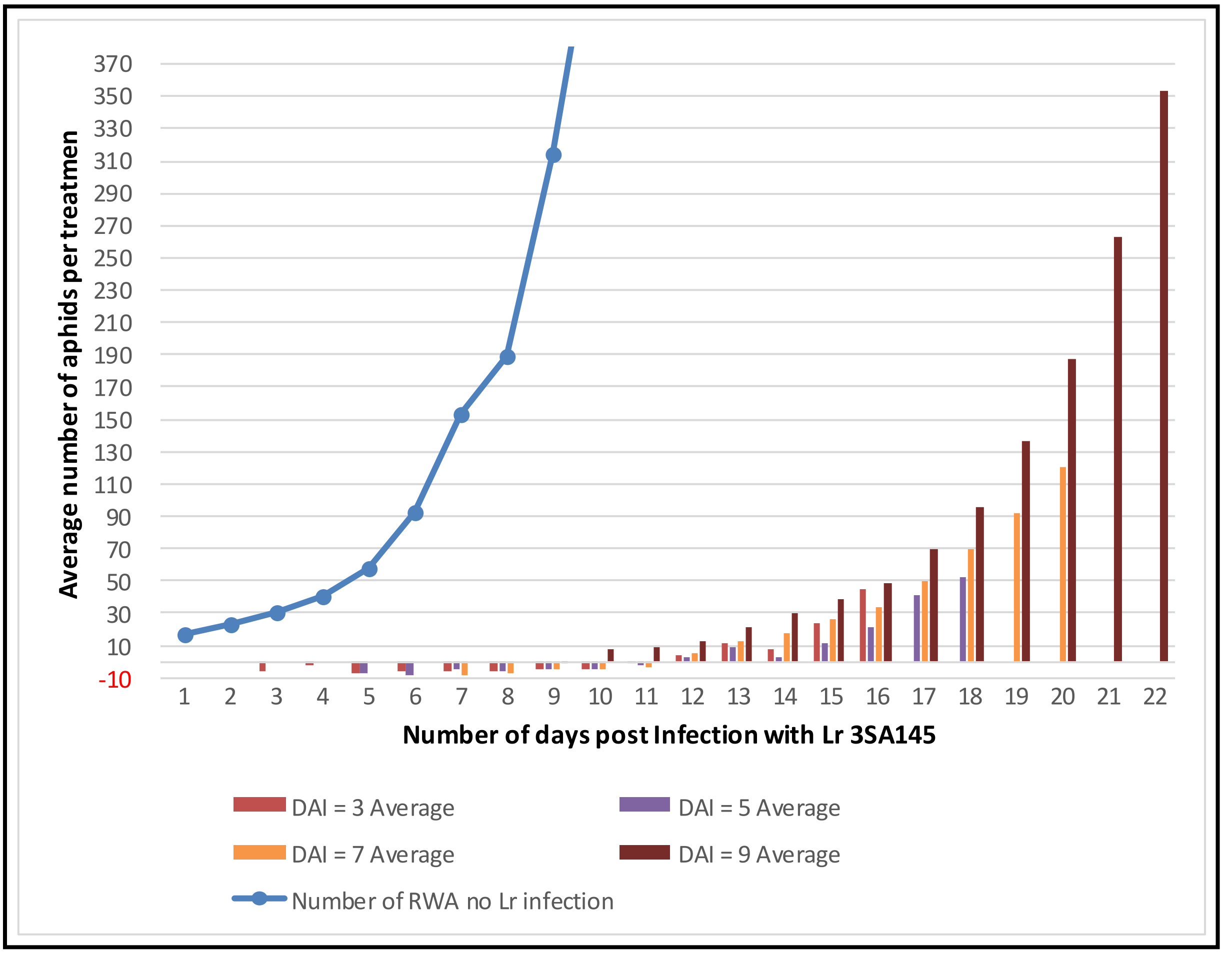


Figure 4.3: RWA SA1 population growth on SST 356 wheat cultivar with and without pre infection with *P. triticina* race 3SA145.

4.4 DISCUSSION

Plants in both natural and cultivated populations defend themselves from a wide range of invaders but this has often not protected them (Dangl and Jones, 2001). For a pathogen to cause disease it has to first overcome the plant's passive defence mechanism mediated by the cuticle of epidermal cells and suberized cell walls which contain cutin and suberin respectively (Freeman and Beattie, 2008). The Russian wheat aphid is a serious pest that affects wheat production throughout the world including South Africa. Host plant resistance is the foundation of cereal insect pest management programs and several *D. noxia* resistance (*Dn*) genes have been introduced into commercial cultivars to manage aphid infestation (Khan *et al*., 2013; Puterka *et al*., 2015). The advantage of resistant cultivars to producers is a direct economic benefit observed by a reduction in the cost of pesticides and fuel owing to fewer applications. The deployment of genetic resources for resistance also assists in achieving yield stability without resorting to harmful chemicals. Russian wheat aphid resistant cultivars have been bred throughout the world for a very long time and are numerous among commercially available cultivars to South African farmers (Tolmay *et al*., 2007). However, the challenge with this system is the development of new aphid biotypes that could overcome this resistance. Breeders and plant pathologists are looking for alternative methods to crop damage by aphids. One of the methods under investigation is the use of biological agents that could prime the plant resistance mechanism before aphid attack. These substances could induce resistance by activating dedicated resistant pathways when challenged by the insect or pathogen.

Two main pathways are induced by plants when challenged by pests and pathogens. The first being hypersensitivity reaction followed by the systemic acquired resistance and the second is induced systemic resistance (Kombrink and Schmelzer, 2001). Induced protection of plants against various pathogens by biotic or abiotic agents is by no means a new phenomenon. It has been reported since 1930s when Chester (1933) proposed the term 'acquired physiological immunity'. After this period several terminologies have been employed to describe the phenomenon of induced resistance such as systemic acquired resistance (Ross, 1961), 'translocated resistance' (Hurbert and Helton, 1967) and plant immunisation (Tuzun and Kuc, 1991). Induced resistance has been defined as an enhancement of the plant's defensive capacity against a broad spectrum of pathogens and pests acquired after appropriate stimulation. The resulting elevated resistance due to an inducing agent upon infection by a pathogen or infestation by insect herbivore is called induced systemic resistance (ISR) or systemic acquired resistance (SAR) (Hammerschmidt and Kuc, 1995). Mansor (2012) demonstrated that priming of plants with potassium phospate conferred resistant to susceptible cultivars and also increased the early response in resistant cultivars. This was further confirmed by the increase expression of defence genes (pal and LOX) and increases in resistance to the Russian wheat aphid based on the phenotypic assessment.

Our results indicated that prior infection of wheat with urenodinospores of leaf rust could induce a defence response and delayed initial infestation of wheat by Russian wheat aphid biotype SA1 by preventing the aphids from colonising the plants. As observed in the control plants, irrespective of the cultivar type (resistant or susceptible), the aphid population increased from the initial dose (10 aphids) placed on the plants, immediately following infestation. The aphids began to reproduce exponentially, however the rate of reproduction was lower in SST 347 when compared to SST 356, confirming the fact that this cultivar is resistant to Russian wheat aphid biotype SA1. This situation was quite different with the plants pre treated with leaf rust. Fewer aphids colonised the previously infected plants as aphids numbers remained below the initial dose of ten on both resistant and susceptible cultivars up to 15/16 days and 12/13 days respectively. With the pre infected resistant cultivar, aphid population only exceeded the initial infestation of 10 aphids 15-16 days after infection with leaf rust whereas this appeared earlier (12-13) days after infection with the moderately resistant aphid and Lr susceptible cultivar.

The response observed in the susceptible cultivar could be likened to the plants normal defence mechanism that was primed and lasted only for a short period of time while in the resistant cultivar the presence of *Dn* gene offered resistance to the aphid biotype. These host plants could be exhibiting a type of resistance known as antixenosis (non-preference) in which the primed host plants deferred aphids from settling and colonising them. The priming resulted in the plant switching on its active defence mechanism in respond to pathogen infection. Reports have showed that following tissue damage, endogenous plant elicitors are released called damaged-associated molecular patterns (DAMPs) that mediate defence response to both pathogens and herbivores (Jones and Danl, 2006; Heil, 2009). This could be the primary reason why the aphids did not colonise the plants initially because the plants active defence was switched on. Plants exposed to various pathogens and environmental stresses can activate this defence that is also dependent on the recognition of the pathogen by the plant (Ton *et al*., 2009). This defence is termed effector-triggered immunity (ETI) with its effectiveness dependent on the rapid recognition of the pathogen-encoded effector protein (avr) by the host resistance ® gene (Jones and Dangl, 2006; Harris *et al*., 2015). ETI occurs rapidly through the oxidative burst, localised cell death (HR), accumulation of phytoalexins, synthesis-related (PR) proteins and cell strengthening proteins (Mur *et al*., 2008; Bendnarek and Osbourn, 2009). HR was observed on the leaves by the appearance of necrotic lesions around the infection sites. HR can also result in the activation of systemic acquired resistance (SAR) in distal uninfected cells that enabled the plants to be resistant for longer periods. Cell wall strengthening following infection has been ascribed to the deposition of callose (a 1, 3-β glucan polymer), different phenolic compounds as well as synthesis of diverse toxic compounds such as antimicrobials (Bendnarek an Osbourn, 2009; Ellinger *et al*., 2013; Voight, 2014); this could have prevented the aphids from probing the plant thus sending them off.

The observed retarded aphid population build up later post infection with leaf rust was an indication of the active defence being switched on within the cultivars during the first two weeks but this was not surprising. Wei *et al*. (1991) reported that the duration of crop protection using pathogens as inducing agents is usually less following the induction as compared to that of plant growth-promoting Rhizobacteria (PGPR)-mediated ISR. The plant switching off its active defence could as well be attributed to the absence of re-infection by the rust pathogen since the controlled environment did not provide the necessary conditions. Therefore the inducing agent was no longer active in the plant to provoke a defence response.

4.5 CONCLUSION

Wheat farmers who have minimal access to chemical inputs and resistant cultivars often achieve very low wheat yields due to biotic challenges from diseases and pests. Improvement of plant basal resistance may be an effective way to protect crop plants against diseases as well as insect pests. The treatment of Russian wheat aphid resistant and moderately resistant wheat cultivars with urediniospores of leaf rust race 3SA145 resulted in systemic protection of the plants against Russian wheat aphid infestation**.** Although our results indicate that prior infection of wheat with leaf rusts delays infestation with Russian wheat aphid in the laboratory, this might not be applicable in the field. Wei *et al*. (1991) reported that the utilisation of pathogenic organisms as inducing agents has not been successful under field conditions. Elicitors of induced responses can be sprayed on crops to build the natural defence system against damage caused by insects. The induced response can also be engineered genetically so that defensive compounds are constitutively produced in plants against herbivore attack. Although some metabolic costs is involved in induced responses (Agrawal *et al*., 2002), they are very important when aimed at eradicating stress of immediate concern, as most of these chemicals are produced in response to herbivore attack (Miranda *et al*., 2007; Steppuhn and Baldwin, 2007). Induced defences make the plants phenotypically plastic, and thereby, decrease the chances of the attacking insects to adapt to the induced chemicals (War *et al*., 2012).

CHAPTER FIVE

GENERAL DISCUSSION

5.1 Introduction

Breeders at SENSAKO and others have introduced wheat cultivars with dual resistance to RWA and leaf rust including Matlabas, Sengu, SST347, Pan 3379, and Pan 3195 (ARC-SGI, production guide, 2015; Terefe *et al*., 2014). No information is available as to whether Russian wheat aphids do not populate wheat plants infected with rusts and vice versa. Reports have shown that the defence mechanism of wheat to RWA is typically a hypersensitive response (HR) induced by RWA infestation (van der Westhuizen *et al*., 1998; 2002; Moloi and van der Westhuizen, 2006). This includes the induction of the intercellular β-1, 3-glucanase, peroxidases and chitinases which are also up regulated as a defence response during pathogen attack. During pathogen attack, the earliest events recognised during HR include the generation of reactive oxygen species (ROS) such as superoxide anion (O_2^-) and hydrogen perioxide (H_2O_2) (Alvarez *et al*., 1988; Bolwell, 1999; Fath *et al*., 2002). This has also been reported to occur in wheat in response to RWA infestation (Van der Westhuizen *et al*., 1998; Moloi and Van der Westhuizen, 2006). It is therefore hypothesised that there is a similar mechanisms of resistance in wheat to RWA and leaf rust. As the actual mechanisms of resistance for either RWA or leaf rust are not known we used a proteomics approach to identify unique proteins up- or down-regulated in response to RWA infestation or leaf rust infection.

5.2 Resistance mechanism/s of wheat to RWA

Pathways that were involved in the resistant mechanism of wheat to RWA SA1 include methionine synthesis and the Calvin cycle. Enzymes such as fructose-1, 6-bisphosphatase and phosphoribulokinase involved in the plants primary metabolism where upregulated. This resulted in the sustainance of the chloroplast integrity as the plant was responding to the damage imposed by the RWA or to the leakage/loss of metabolites as a result of aphid feeding. Adenosylhomocysteinase, an enzyme involved in methionine synthesis was also involed in the resistance mechanism of wheat to aphid. Methionine is involved in the biosynthesis of the phytohormone ethylene. Ethylene is involved in several defence responses such as xylem occlusions, cell wall-stregnthenining by the production of hydroxyproline-rich glycoproteins, phytoalexins and the induction of PR proteins (Adie *et al*., 2007; Coll *et al*., 2011). This is a HR response characterised by localised cell death in close proximity to the infected site thus making the wheat plant to survive RWA infestations.

5.3 Resistance mechanism/s of wheat to leaf rust

Pathways that were involved in the resistant mechanism of wheat to leaf rust include purine biosynthesis and reactive oxygen species (ROS) degradation. Adenylosuccinate synthatase, an enzyme that plays an important role in purine biosynthesis was involved in the resistance mechanism of wheat to leaf rust was upregulated. This pathway is involed in energy metabolism supplying more energy to the infected plant. Catalase, an enzyme known to degrade ROS that activates programmed cell death associated with immune response such as HR responses that clear pathogen and destroys infected cells (Mur *et al.*, 2008). This resistance response helped the wheat plant to survive leaf rust infection.

5.4 Synchronicity of the mechanism of wheat resistance to leaf rust and Russian wheat aphid

This study revealed similarities (synchronicity) in wheat resistance to leaf rust and the Russian wheat aphid. Common Enzymes up regulated in wheat cultivars in response to stress either imposed by RWA SA1 feeding or leaf rust race 3SA145 included key enzymes in photosynthesis such as photosystem II proteins and cytochrome as well as s-adenosylmethionine synthase involved in ethylene pathway. The photosynthetic proteins are involved in maintaining the integrity of the chloroplasts and photosynthetic systems, suggesting that the maintenance of photosynthesis is a key step in the resistance of SST 347 wheat plants to RWA SA1 and leaf rust. The generation of ethylene by the wheat plant is a hypersensitivity reaction (HR), known to protect the plant from pathogen/insect attacks.

Gatehouse (2002) and Botha *et al*. (2014) reported that *D. noxia* differs from many other insect pests in their mode of feeding in that they do not cause major tissue damage or induce plant wounding responses since they feed from plant phloem by inserting a stylet between the cells. Consequently, plant responses to aphid feeding have been reported to be similar to those induced by pathogen attack (Smith and Boyko, 2007). This could be the reason why we identified similar proteins that were up-regulated in stressed wheat plants in response to aphid feeding and leaf rust infection. Some studies have reported that the early plant responses to aphid or pathogen infection, share common events such as phosphorylation, membrane depolarization , calcium influx and release of reactive oxygen species (ROS) (Garcia-Burgger *et al*., 2006), thereby leading to the activation of phytohormone-dependent pathways. These are followed by ethylene and jasmonate dependent responses (Maffie *et al*, 2007a & b).

http://entoweb.okstate.edu/ddd/insects/russianwheataphid.htm **http://www.mississippi-crops.com/2013/02/28/wheat-leaf-rust-detected-in-mississippi/**

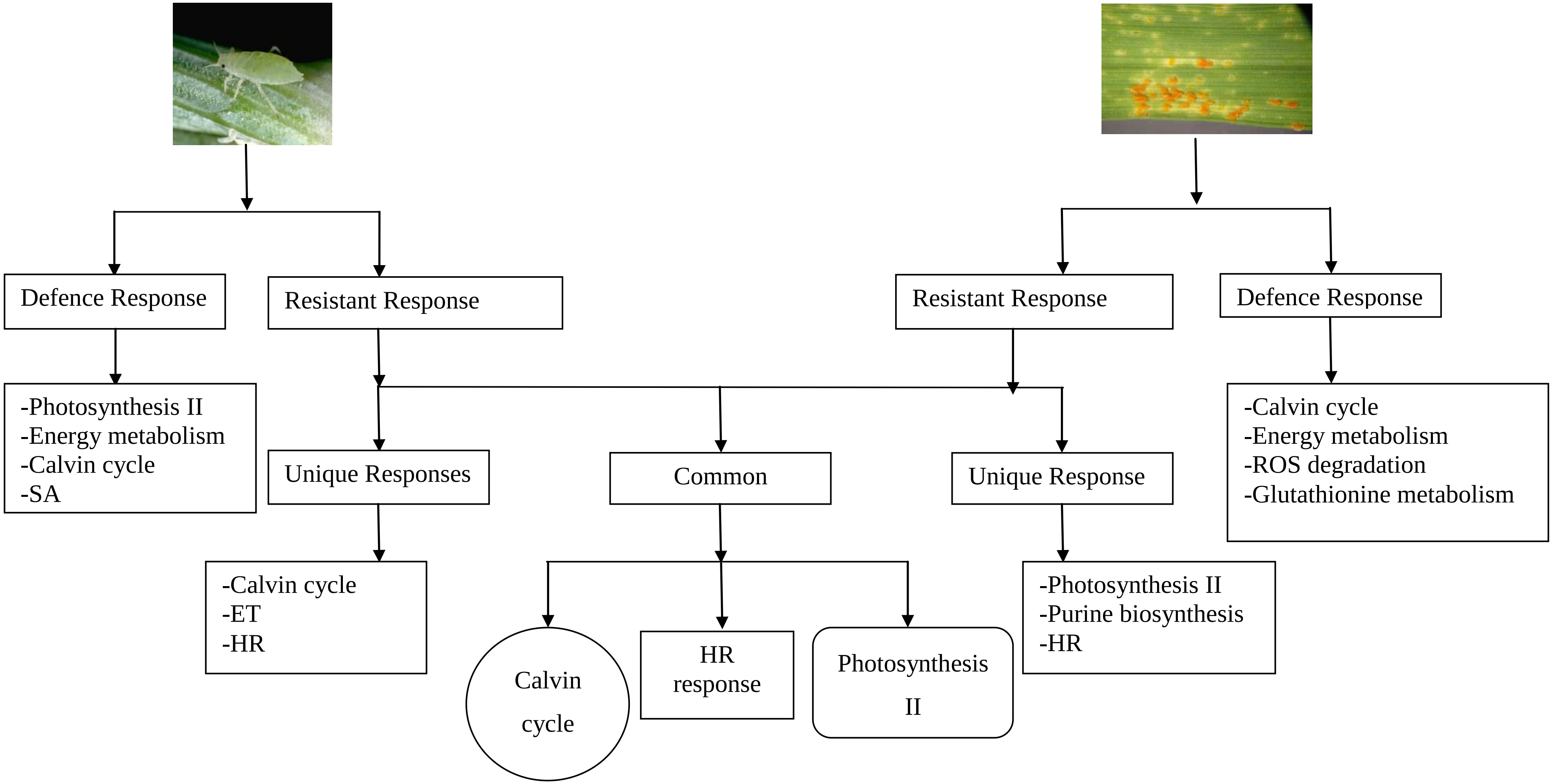


Figure 5.1: Proposed model

5.5 Future work

In order to validate the model above, we plan to carry the following experiments.

5.5.1 Western blotting/ELISA

The up-regulating of the specific key regulating proteins in the identified resistance mechanism will be confirmed at a protein level using antibodies generated against these specific proteins using western blots or ELISA of plant extracts infested with RWA or infected with LR at different time intervals.

5.5.2 qPCR

The up-regulation or transcription of specific key gene regulating the pathways identified in this study will be monitored using qPCR.

5.5.3 Adding elicitors

A susceptible wheat cultivar will be taken and treated with various elicitors such as JA, SA or ABA and resistance to RWA and leaf rust will be scored. This will give an idea if the pathway was common to both leaf rust infection and aphid infestation. If the protein was only expressed in RWA infested plants, it should become RWA resistance and not LR. The deposition of callose will also be evaluated using fluorescent microscopy.

5.5.4 Measuring particular enzyme activity

Enzymes within the common pathways and unique pathways identified in this study will be selected and the enzymatic activity will be monitored in resistant cultivars to RWA or LR at different time frames.

5.5.5 Measuring role of Ca^{2+} in the response mechanism

Confocal microscopy will be used to monitor Ca^{2+} fluxes in resistant and susceptible cultivars at various time frames, in response to RWA infestation or LR infection.

5.5.6 Identification of kinases or phosphorylated proteins

Antibodies to specific kinases will be generated to identify them and their activities measured or phosphorylated proteins will be identified and quantitate by liquid chromatography mass spectrometry (LC/MS).

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APPENDIX A: REAGENTS PREPARATIONS

i) ReadyPrep 2-DE Rehydration/sample buffer 1

- ❖ Add 5.6 ml of ReadyPrep proteomic water to the lyophilised vial and swirl gently until the contents are completely dissolved
    - o The solution can be warm slightly in the palm of the hand or in a water bath to speed the dissolution process
    - o After reconstitution, each vial contains 10mL of 7 M urea, 2 M thiourea, 1%(w/v) ASB-14 detergent, 40 mM Tris base and 0.001% bromophenol blue
    - o Any unused reconstituted ReadyPrep 2-D rehydration/sample buffer 1 should be stored frozen at -80 ^{o}C in 1 to 2 mL aliquots.

ii) ReadyPrep TBN Reducing Agent

- ❖ Open the ampoule by snapping the top off at the scored neck and transfer the entire contents of the ampoule into the empty screw-cap storage vial provided, screw down the cap tightly and store at -20 ^{o}C to prevent evaporation of the TBP
    - o *1 ampoule contain 0.6 mL of 200 nmM tributylphosphine (TBP) in 1-methyl-2-pyrrolidinone sealed under nitrogen gas*

1. Prepare 3-5 dilutions of protein standard from 0.2 mg/mL to 1.5 mg/mL.

NB. A standard curve should be prepared each time the assay is performed and for best results, the standards should always be prepared in the same buffer as the sample.

i) Preparation of Bovine serum albumin (BSA) stock (2 mg/mL)

0.002 g of powder in 1000 µL of DH_2O
X5 X5
= 0.01 g of powder in 5000 µL of DH_2O

Dilution of BSA for the standard curve

1 mg/mL = 1 mL of 2 mg/mL + 1 mL of DH_2O

0.75 mg/mL = 0.75 mL of the 1 mg/mL dilution + 0.25 mL of DH_2O

0.5 mg/mL = 0.5 mL of the 0.75 mg/mL dilution + 0.5 mL of DH_2O

0.25 mg/mL = 0.5 mL of the 0.5 mg/mL dilution + 0.5 mL of DH_2O

0.125 mg/mL = 0.5 mL of the 0.25 mg/mL dilution + 0.5 mL of DH_2O

0.0625 mg/mL = 0.5 mL of the 0.125 mg/mL dilution+ 0.5 mL of DH_2O

0.03123 mg/mL = 0.5 mL of the 0.0625 mg/mL dilution + 0.5 mL of DH_2O

Dilution of protein samples

1:1 = 10 µL of protein + 10 µL of DH2O

1:10 = 10 µL of the 1:1 dilution + 90 µL of DH_2O

1:100 = 10 µL of the 1:10 dilution + 90 µL of DH_2O

1:1000 =10 µL of the 1:100 dilution + 90 µL of DH_2O

Preparartion of 1X SDS-PAGE gel running Buffer

- Prepare a 1x Tris/glycine/SDS(TGS) running buffer by adding 100 mL of the 10x TGS to 900 mL of DH_2O

OR

- Prepare the 1x TGS from TGS powders as follows:

(25 mM Tris, 192 mM glycine, 0.1% SDA, pH 8.3)

Tris Base	3.03 g
Glycine	14.4 g
SDS	1.0 g
Water	1 L, pH not adjusted

Preparation of Equilibration buffer I and II

NB The equilibration buffers should be prepared about 15 minutes before use. If the IPG strips were at -80 ^{o}C they can be removed and placed on the lab bench top to thaw at this time. The strips require 10-15 minutes to thaw. It is best to not leave the thawed IPG strips for longer than 15-20 minutes as diffusion of the proteins can result in reduced sharpness of the protein spots.

Equilibration buffer I

- To each one bottle of the buffer carefully add 13.35 mL of the supplied 30% glycerol solution. Each bottle contains stirbar. Place the bottle onto a stirplate and mix until all the solids have completely dissolved. It may be necessary to periodically swirl the contents of the bottle to dislodge solids remaining on the walls of the glass bottle. The solids generally will dissolve in less than 5 minutes. The bottle will chill as the urea in the solids dissolves. To expedite this buffer reconstitution process the bottle can be warmed slightly in the palm of the hand or placed into a water bath set for 25-30 ^{o}C as the solution stirs. Do not heat above 30 ^{o}C**.**

Equibration bufferII

- To each bottle of the buffer carefully add 13.35 mL of the supplied 30% glycerol solution. Each bottle contains a stirbar. Place the bottle onto a stirplate and mix until all the solids have completely dissolved as described above for equilibration buffer I.

Preparation of 50 mm NH_4HCO_3

- 0.2 g NH_4HCO_3 was dissolved in 50 mL in H_2O (NH_4HCO_3 Mw=79.06 g/mol). The pH checked (it should between 7.5-8.5) and solution stored at 4 ^{o}C and used within 2 weeks.

Preparation of 5.5 mM $CaCl_2$ in 25 mm NH_4HCO_3

- 0.1 g NH_4HCO_3 per 50 mL H_2O + 0.03 g $CaCl_2$ (Mw=110.98 g/mol) stored at 4 °C

Preparation of 50 mM NH_4HCO_3/50% MeOH (50 mL) (stored at 4 °C and used within 2 weeks)

- 0.2 g NH_4HCO_3 to 25 mL of MS grade H_2O +25 mL of 100% MeOH

Preparation of 25 mM NH_4HCO_3 in 50% ACN (2mL) (stored at 4 °C and used within two weeks)

- 1000 µL of 50 mM NH_4HCO_3 in MS-grade H_2O + 1000 µL of 100% ACN

Preparation of 75% ACN (10 mL) (stored at 4 °C and used within two weeks)

- 2.5 mL MS-grade H_2O + 7.5 mL ACN

Preparation of Trypsin Stock

- 200 µL of the buffer supplied in the kit added to one vial containing 20 µg trypsin and placed on ice
- the vial vortexed to dissolve the trypsin
- 20 µL aliquots in 0.5 mL eppendorfs (work rapidly in an ice bath)
- The aliquots immediately stored at -20 °C

Preparation of fresh 10 ng/µL trypsin

- 20 µL stock trypsin+180 µL, 5.5 mM $CaCl_2$ in 25 mM NH_4HCO_3 in milliQ-H_2O

Preparation of fresh Stock (1 mL) of 1 M DTT

- 0.154 g DTT dissolved in 1000 µL of milliQ-H_2O

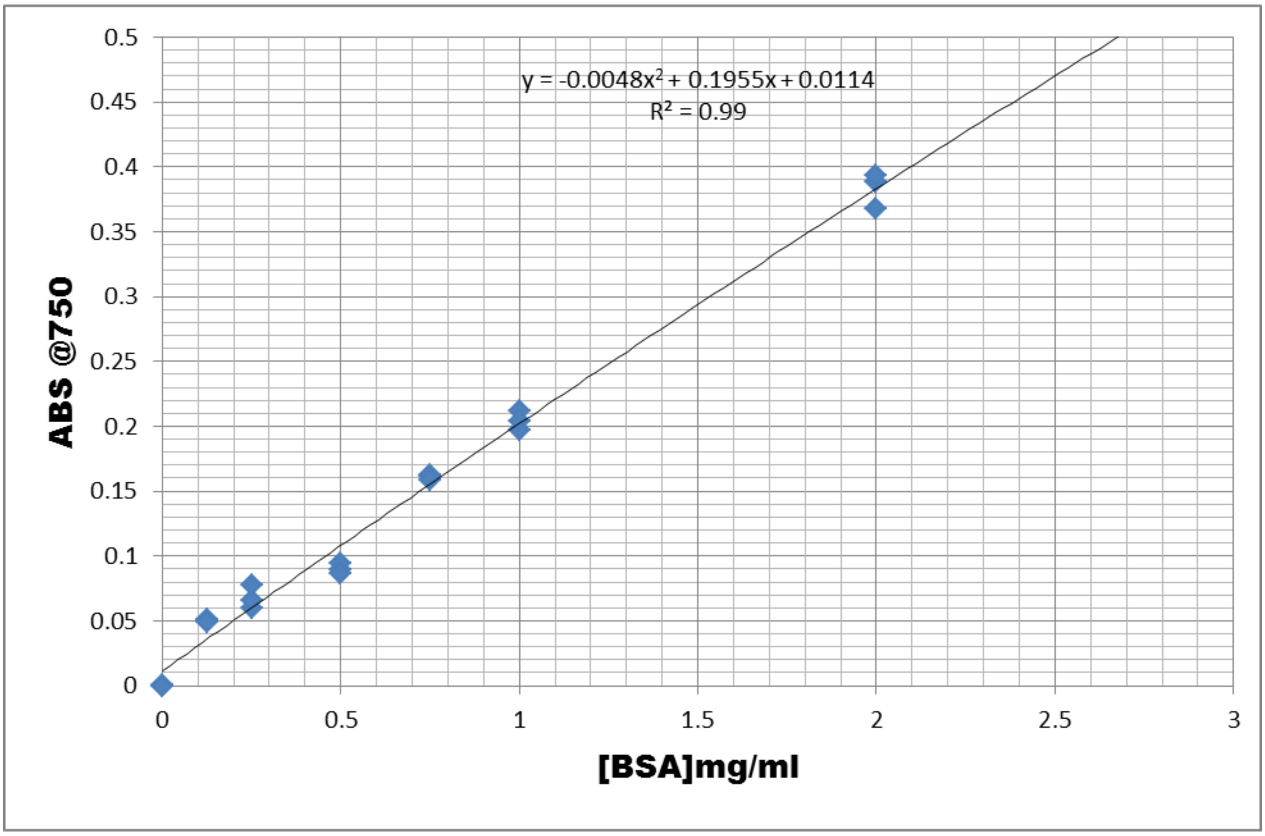
Preparation of fresh 10 mM DTT in 25 mM NH_4HCO_3

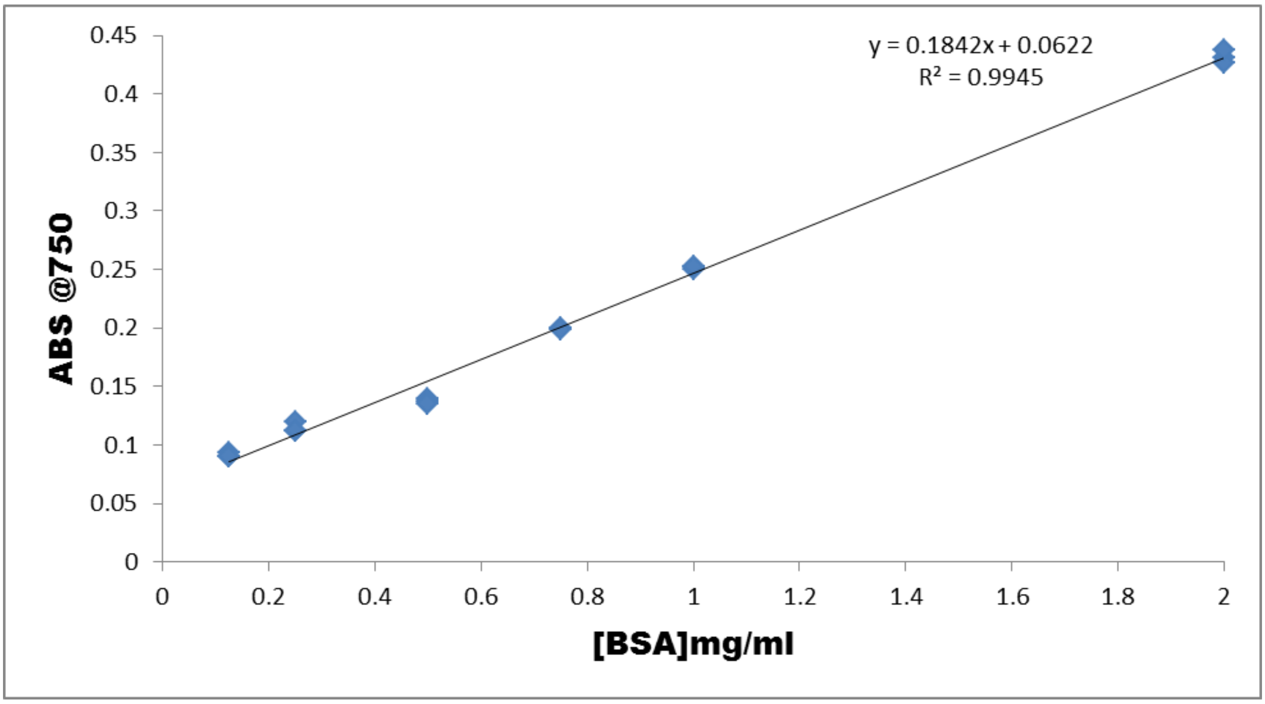
- 10 µL of 1M DTT + 495 µL of 50 mM NH_4HCO_3 + 495µL MS-grade H_2O

Preparation of 55 mm IAA in 25 mM NH_4HCO_3 (1 mL) (stored in a dark container)

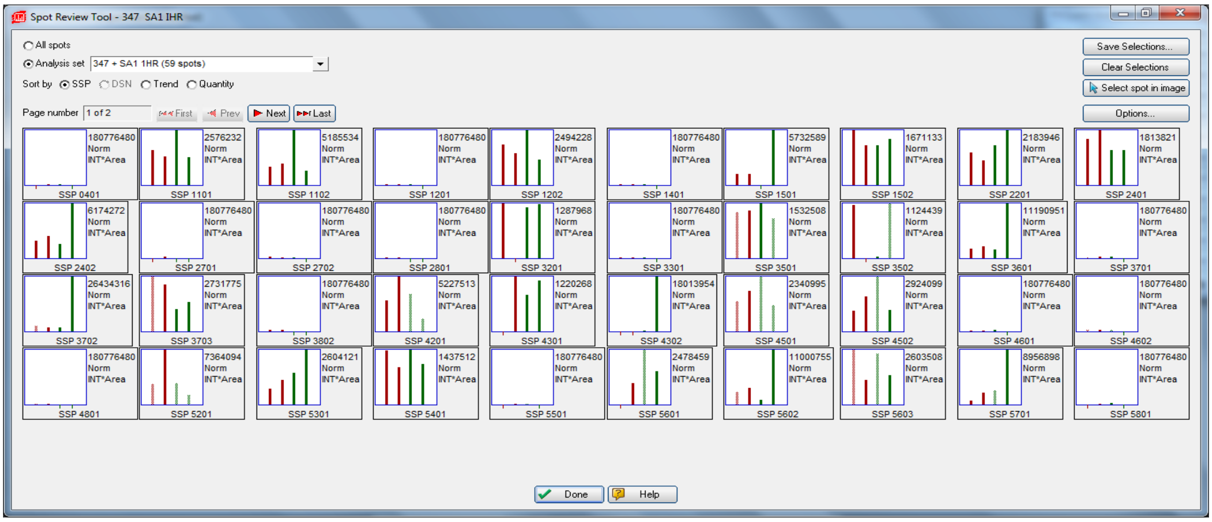
- 0.010 g IAA was weighed and 500 µL 50 mM NH_4HCO_3 + 500 µL MS-grade –H_2O added

APPENDIX B: BSA STANDARD CURVES



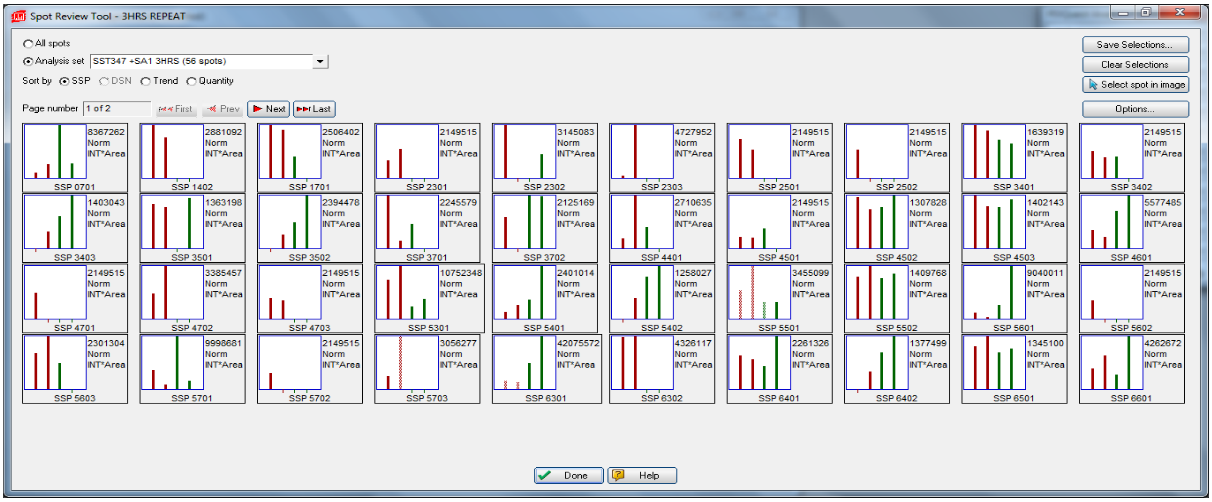


APPENDIX C: RESULTS OF ANALYSIS OF DIFFERENTIALLY EXPRESSED PROTEINS WITH PDQUEST™ BASIC SOFTWARE VERSION 8.01: QUANTITY GRAPH REPORTS



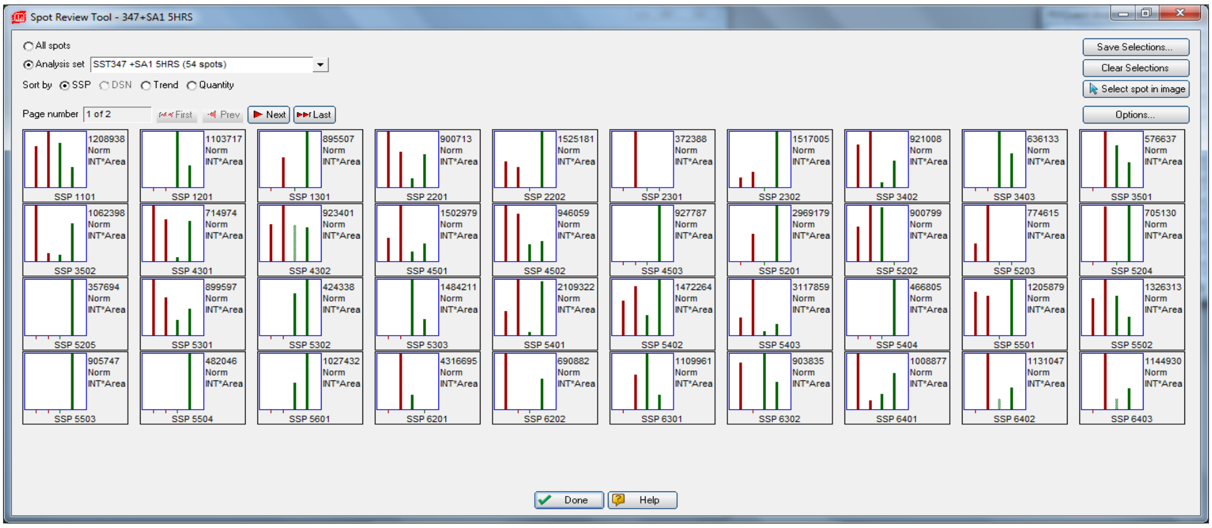
Spot Review Tool - 347 SA1 IHR

○ All spots
● Analysis set 347 + SA1 1HR (59 spots)
Sort by ● SSP ○ DSN ○ Trend ○ Quantity
Page number 1 of 2



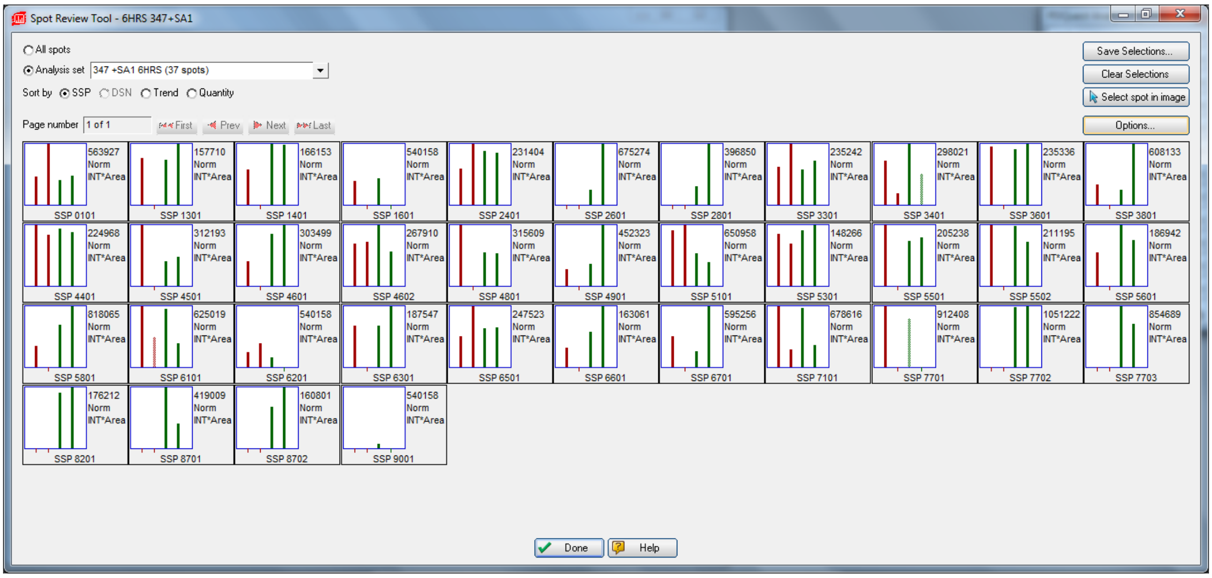
Spot Review Tool - 3HRS REPEAT

○ All spots
● Analysis set SST347 +SA1 3HRS (56 spots)
Sort by ● SSP ○ DSN ○ Trend ○ Quantity
Page number 1 of 2

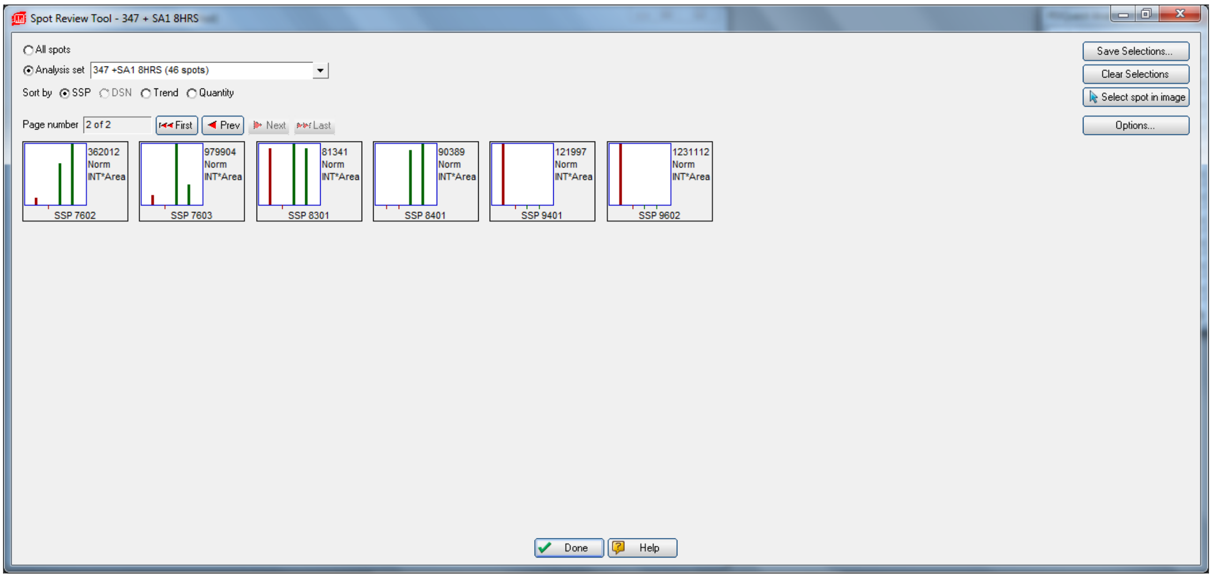


Spot Review Tool - 347+SA1 5HRS

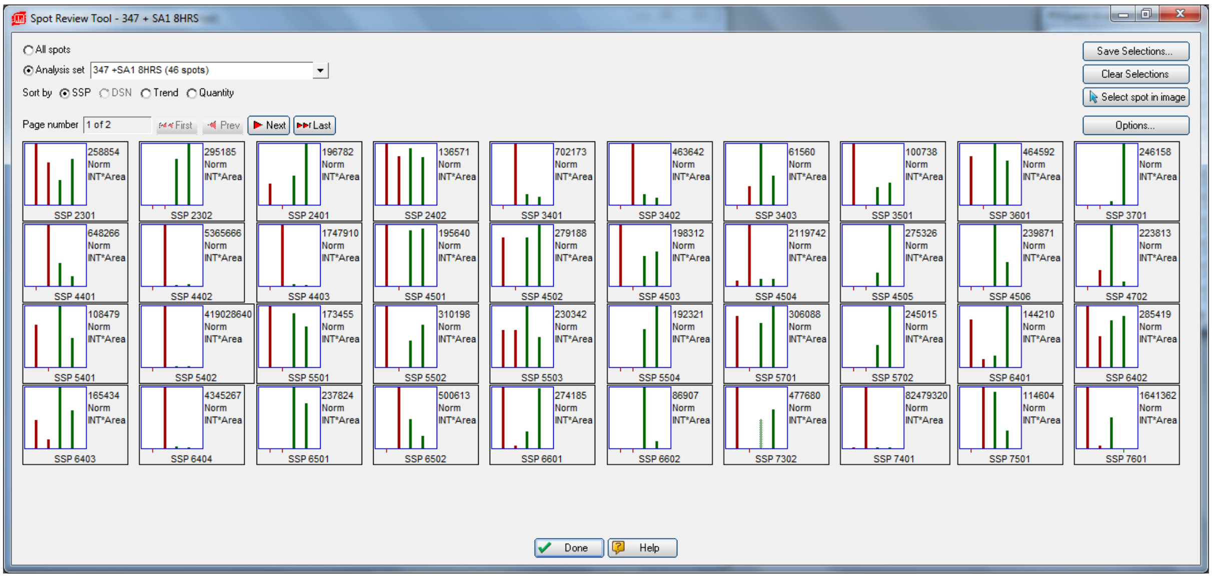
○ All spots
● Analysis set SST347 +SA1 5HRS (54 spots)
Sort by ● SSP ○ DSN ○ Trend ○ Quantity
Page number 1 of 2


Spot Review Tool - 6HRS 347+SA1

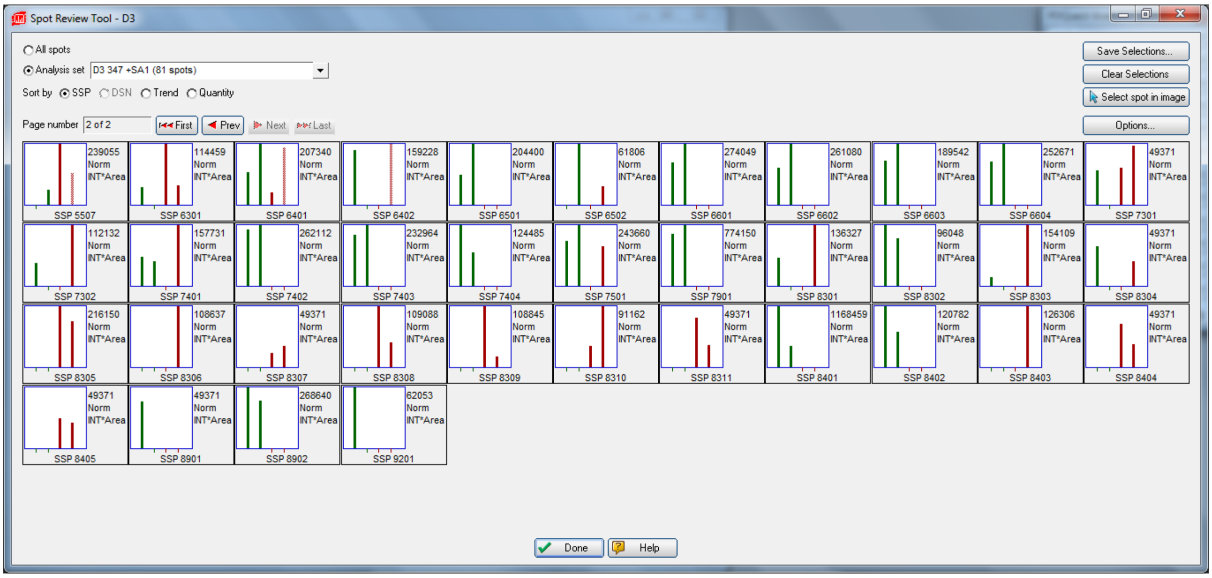
○ All spots
● Analysis set: 347 +SA1 6HRS (37 spots)
Sort by: ● SSP ○ DSN ○ Trend ○ Quantity
Page number: 1 of 1


Spot Review Tool - 347 + SA1 8HRS

○ All spots
● Analysis set: 347 +SA1 8HRS (46 spots)
Sort by: ● SSP ○ DSN ○ Trend ○ Quantity
Page number: 2 of 2


Spot Review Tool - 347 + SA1 8HRS

○ All spots
● Analysis set: 347 +SA1 8HRS (46 spots)
Sort by: ● SSP ○ DSN ○ Trend ○ Quantity
Page number: 1 of 2


Spot Review Tool - D3

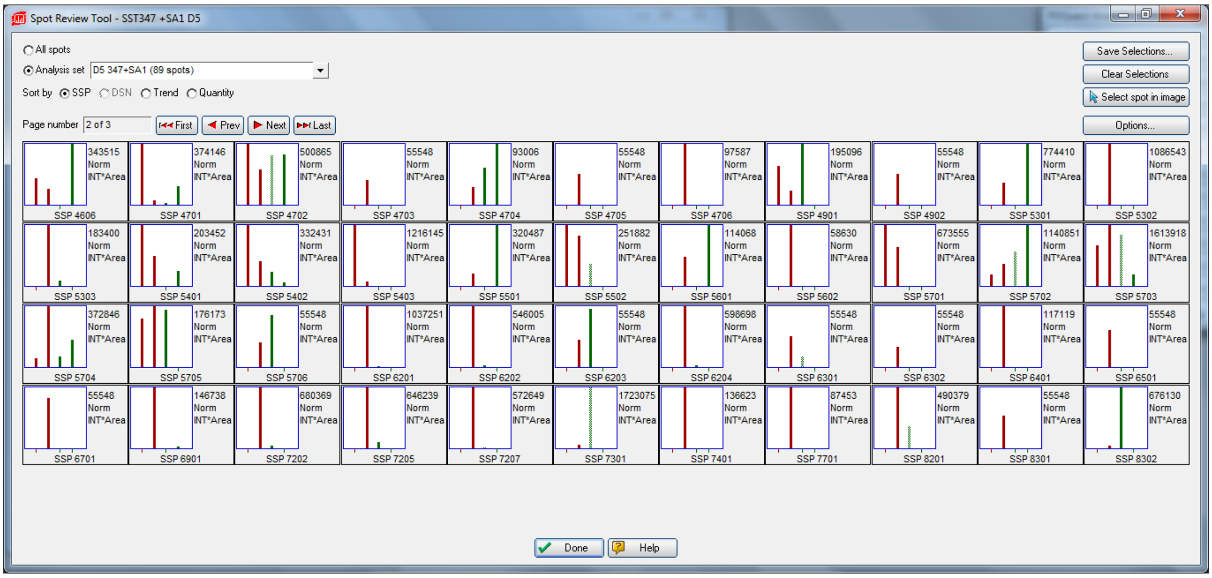
○ All spots

● Analysis set D3 347 +SA1 (81 spots)

Sort by ● SSP ○ DSN ○ Trend ○ Quantity

Page number 2 of 2

239055 Norm INT*Area SSP 5507	114459 Norm INT*Area SSP 6301	207340 Norm INT*Area SSP 6401	159228 Norm INT*Area SSP 6402	204400 Norm INT*Area SSP 6501	61806 Norm INT*Area SSP 6502	274049 Norm INT*Area SSP 6601	261080 Norm INT*Area SSP 6602	189542 Norm INT*Area SSP 6603	252671 Norm INT*Area SSP 6604	49371 Norm INT*Area SSP 7301
112132 Norm INT*Area SSP 7302	157731 Norm INT*Area SSP 7401	262112 Norm INT*Area SSP 7402	232964 Norm INT*Area SSP 7403	124485 Norm INT*Area SSP 7404	243660 Norm INT*Area SSP 7501	774150 Norm INT*Area SSP 7901	136327 Norm INT*Area SSP 8301	96048 Norm INT*Area SSP 8302	154109 Norm INT*Area SSP 8303	49371 Norm INT*Area SSP 8304
216150 Norm INT*Area SSP 8305	108637 Norm INT*Area SSP 8306	49371 Norm INT*Area SSP 8307	109088 Norm INT*Area SSP 8308	108845 Norm INT*Area SSP 8309	91162 Norm INT*Area SSP 8310	49371 Norm INT*Area SSP 8311	1168459 Norm INT*Area SSP 8401	120782 Norm INT*Area SSP 8402	126306 Norm INT*Area SSP 8403	49371 Norm INT*Area SSP 8404
49371 Norm INT*Area SSP 8405	49371 Norm INT*Area SSP 8901	268640 Norm INT*Area SSP 8902	62053 Norm INT*Area SSP 9201							


Spot Review Tool - SST347 +SA1 D5

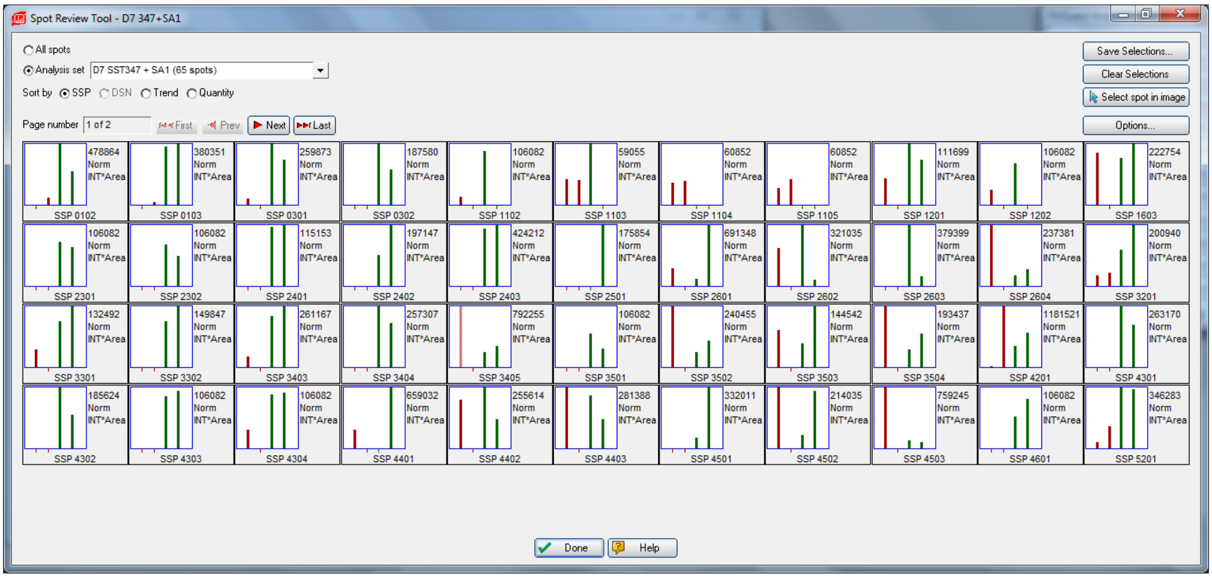
○ All spots

● Analysis set D5 347+SA1 (89 spots)

Sort by ● SSP ○ DSN ○ Trend ○ Quantity

Page number 2 of 3

343515 Norm INT*Area SSP 4606	374146 Norm INT*Area SSP 4701	500865 Norm INT*Area SSP 4702	55548 Norm INT*Area SSP 4703	93006 Norm INT*Area SSP 4704	55548 Norm INT*Area SSP 4705	97587 Norm INT*Area SSP 4706	195096 Norm INT*Area SSP 4901	55548 Norm INT*Area SSP 4902	774410 Norm INT*Area SSP 5301	1086543 Norm INT*Area SSP 5302
183400 Norm INT*Area SSP 5303	203452 Norm INT*Area SSP 5401	332431 Norm INT*Area SSP 5402	1216145 Norm INT*Area SSP 5403	320487 Norm INT*Area SSP 5501	251882 Norm INT*Area SSP 5502	114068 Norm INT*Area SSP 5601	58630 Norm INT*Area SSP 5602	673555 Norm INT*Area SSP 5701	1140851 Norm INT*Area SSP 5702	1613918 Norm INT*Area SSP 5703
372846 Norm INT*Area SSP 5704	176173 Norm INT*Area SSP 5705	55548 Norm INT*Area SSP 5706	1037251 Norm INT*Area SSP 6201	546005 Norm INT*Area SSP 6202	55548 Norm INT*Area SSP 6203	598698 Norm INT*Area SSP 6204	55548 Norm INT*Area SSP 6301	55548 Norm INT*Area SSP 6302	117119 Norm INT*Area SSP 6401	55548 Norm INT*Area SSP 6501
55548 Norm INT*Area SSP 6701	146738 Norm INT*Area SSP 6901	680369 Norm INT*Area SSP 7202	646239 Norm INT*Area SSP 7205	572649 Norm INT*Area SSP 7207	1723075 Norm INT*Area SSP 7301	136623 Norm INT*Area SSP 7401	87453 Norm INT*Area SSP 7701	490379 Norm INT*Area SSP 8201	55548 Norm INT*Area SSP 8301	676130 Norm INT*Area SSP 8302


Spot Review Tool - D7 347+SA1

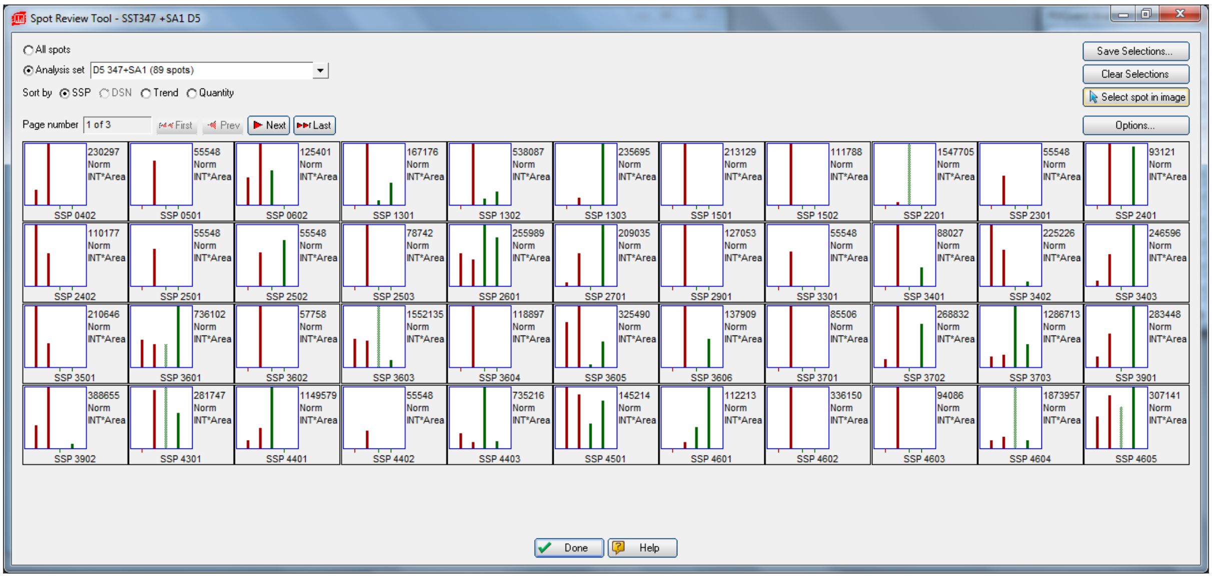
○ All spots

● Analysis set D7 SST347 + SA1 (65 spots)

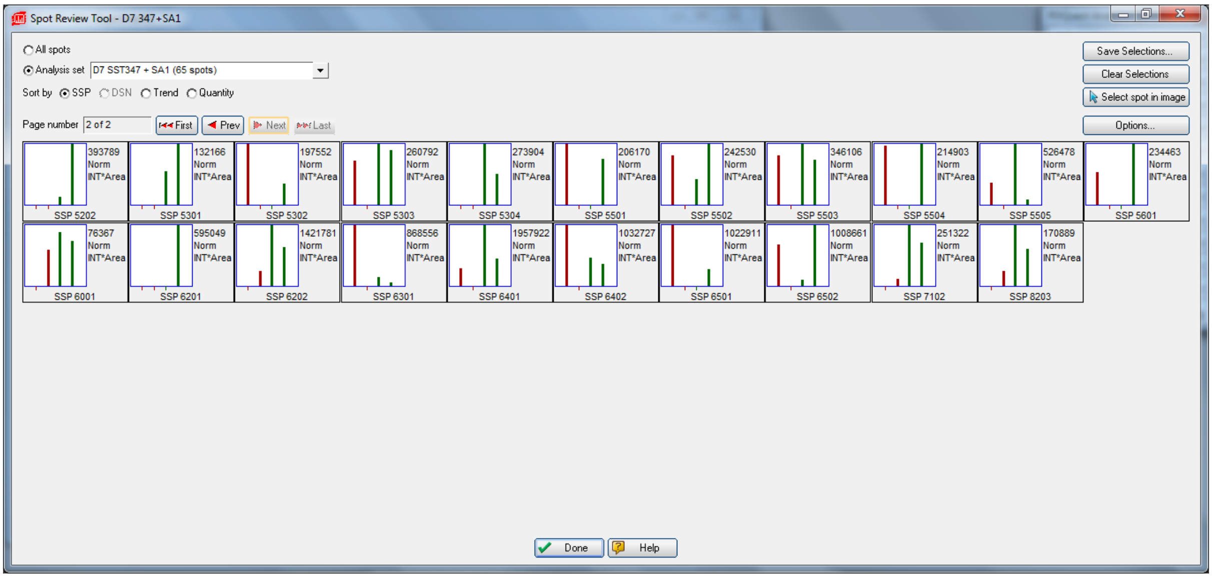
Sort by ● SSP ○ DSN ○ Trend ○ Quantity

Page number 1 of 2

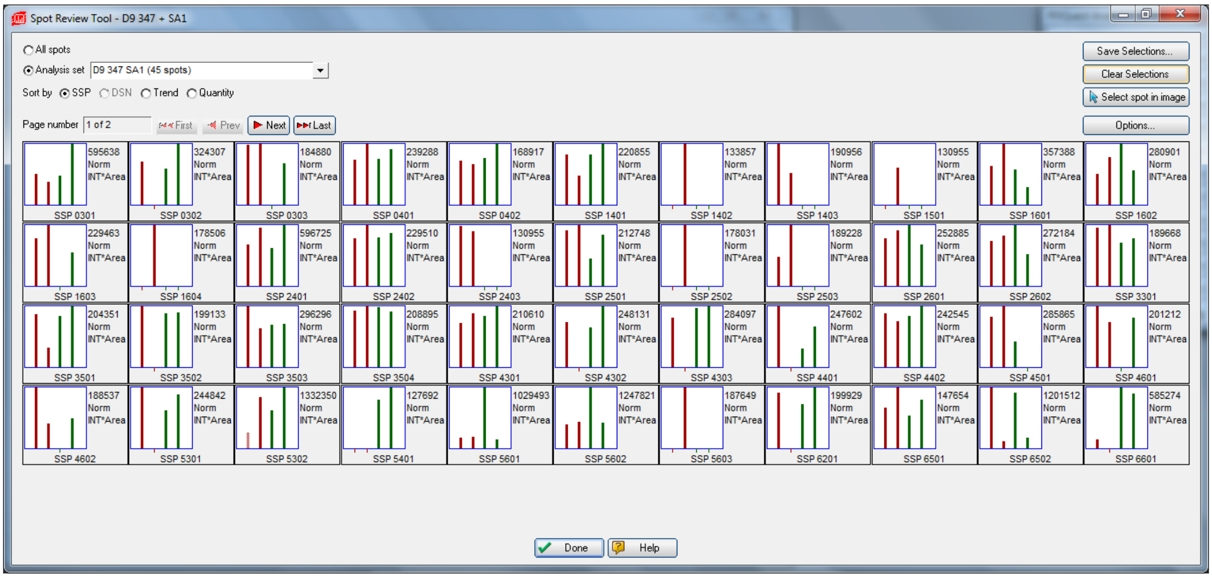
478864 Norm INT*Area SSP 0102	380351 Norm INT*Area SSP 0103	259873 Norm INT*Area SSP 0301	187580 Norm INT*Area SSP 0302	106082 Norm INT*Area SSP 1102	59055 Norm INT*Area SSP 1103	60852 Norm INT*Area SSP 1104	60852 Norm INT*Area SSP 1105	111699 Norm INT*Area SSP 1201	106082 Norm INT*Area SSP 1202	222754 Norm INT*Area SSP 1603
106082 Norm INT*Area SSP 2301	106082 Norm INT*Area SSP 2302	115153 Norm INT*Area SSP 2401	197147 Norm INT*Area SSP 2402	424212 Norm INT*Area SSP 2403	175854 Norm INT*Area SSP 2501	691348 Norm INT*Area SSP 2601	321035 Norm INT*Area SSP 2602	379399 Norm INT*Area SSP 2603	237381 Norm INT*Area SSP 2604	200940 Norm INT*Area SSP 3201
132492 Norm INT*Area SSP 3301	149847 Norm INT*Area SSP 3302	261167 Norm INT*Area SSP 3403	257307 Norm INT*Area SSP 3404	792255 Norm INT*Area SSP 3405	106082 Norm INT*Area SSP 3501	240455 Norm INT*Area SSP 3502	144542 Norm INT*Area SSP 3503	193437 Norm INT*Area SSP 3504	1181521 Norm INT*Area SSP 4201	263170 Norm INT*Area SSP 4301
185624 Norm INT*Area SSP 4302	106082 Norm INT*Area SSP 4303	106082 Norm INT*Area SSP 4304	659032 Norm INT*Area SSP 4401	255614 Norm INT*Area SSP 4402	281388 Norm INT*Area SSP 4403	332011 Norm INT*Area SSP 4501	214035 Norm INT*Area SSP 4502	759245 Norm INT*Area SSP 4503	106082 Norm INT*Area SSP 4601	346283 Norm INT*Area SSP 5201


Spot Review Tool - SST347 +SA1 D5

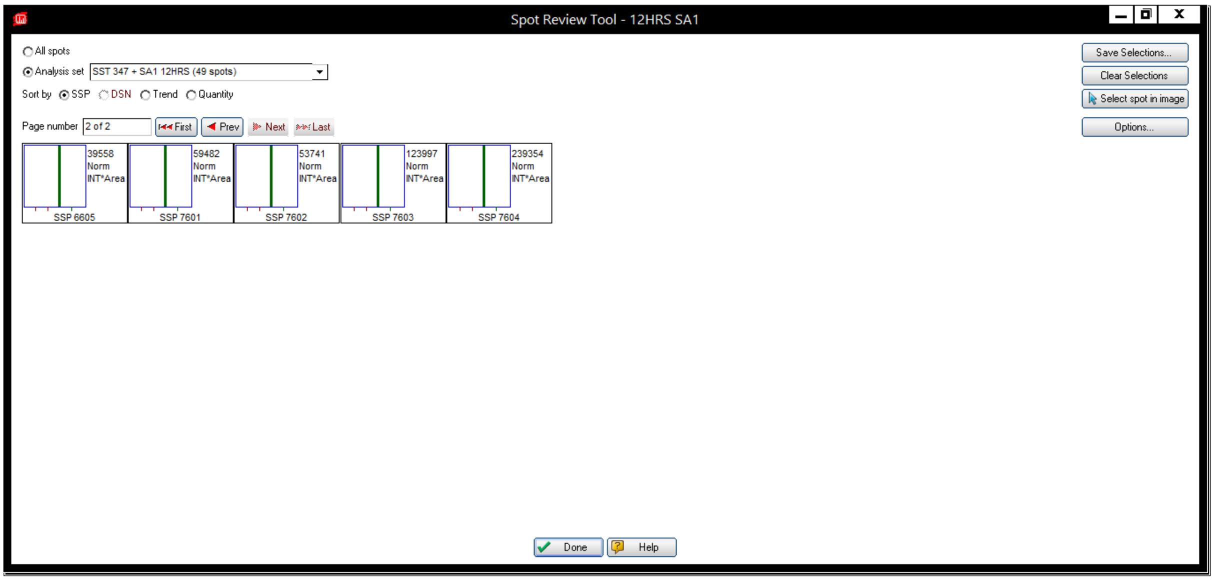
○ All spots
● Analysis set: D5 347+SA1 (89 spots)
Sort by: ● SSP ○ DSN ○ Trend ○ Quantity
Page number: 1 of 3


Spot Review Tool - D7 347+SA1

○ All spots
● Analysis set: D7 SST347 + SA1 (65 spots)
Sort by: ● SSP ○ DSN ○ Trend ○ Quantity
Page number: 2 of 2


Spot Review Tool - D9 347 + SA1

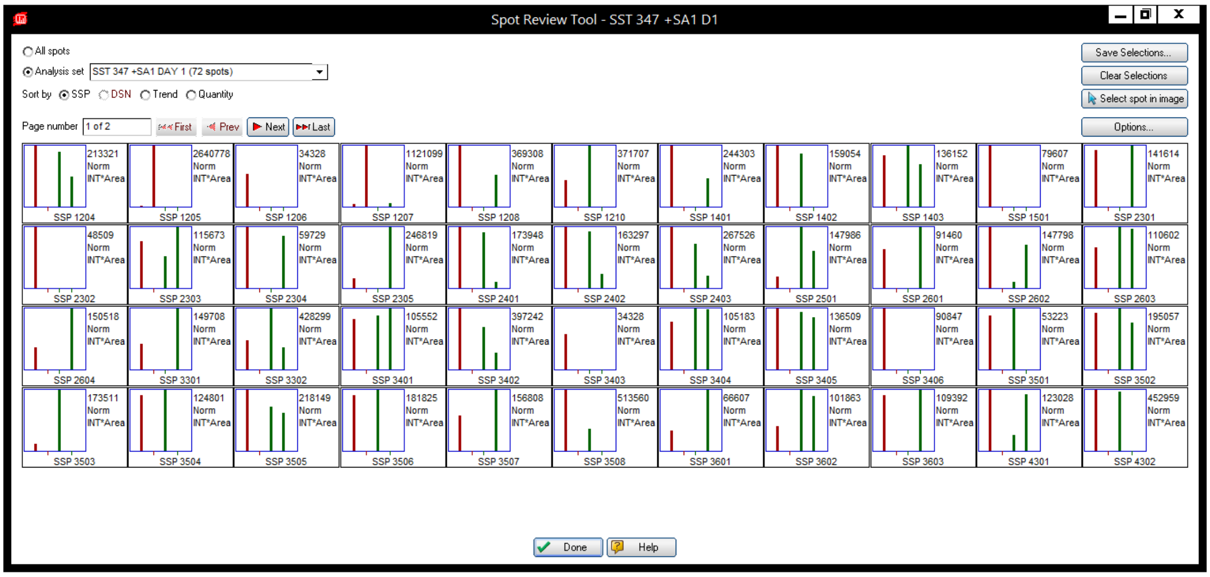
○ All spots
● Analysis set: D9 347 SA1 (45 spots)
Sort by: ● SSP ○ DSN ○ Trend ○ Quantity
Page number: 1 of 2



Spot Review Tool - 12HRS SA1

○ All spots
● Analysis set: SST 347 + SA1 12HRS (49 spots)
Sort by ● SSP ○ DSN ○ Trend ○ Quantity
Page number: 2 of 2

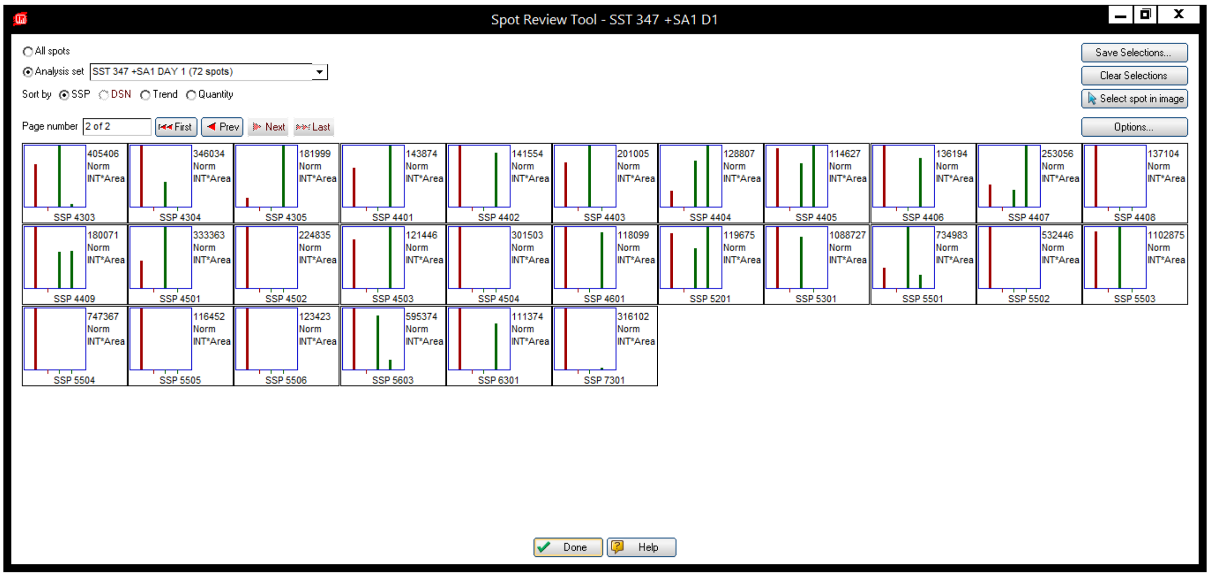
39558 Norm INT*Area SSP 6605	59482 Norm INT*Area SSP 7601	53741 Norm INT*Area SSP 7602	123997 Norm INT*Area SSP 7603	239354 Norm INT*Area SSP 7604



Spot Review Tool - SST 347 +SA1 D1

○ All spots
● Analysis set: SST 347 +SA1 DAY 1 (72 spots)
Sort by ● SSP ○ DSN ○ Trend ○ Quantity
Page number: 1 of 2

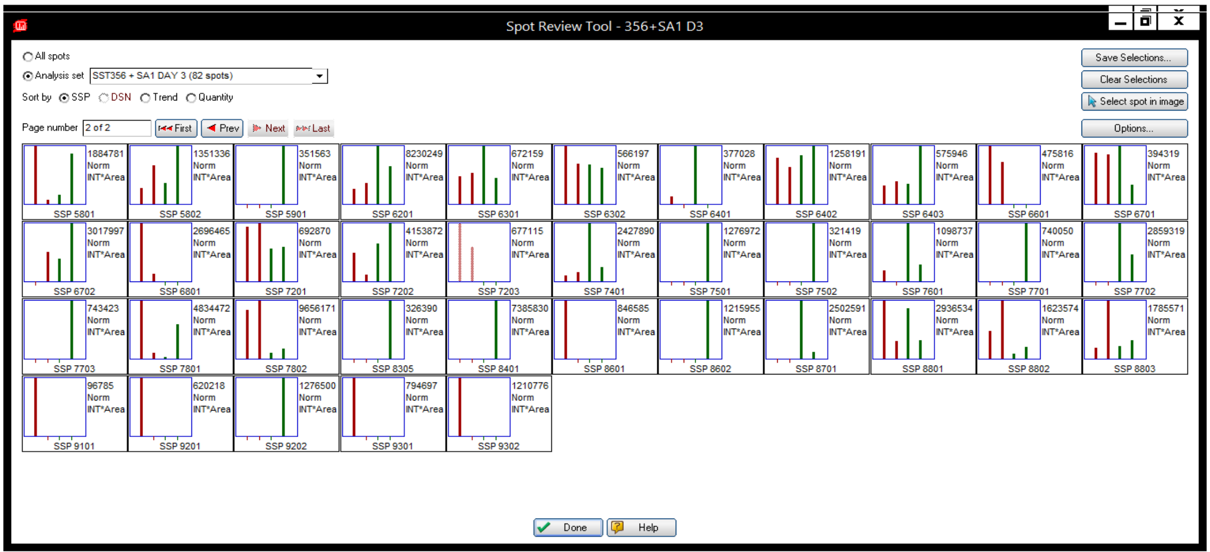
213321 Norm INT*Area SSP 1204	2640778 Norm INT*Area SSP 1205	34328 Norm INT*Area SSP 1206	1121099 Norm INT*Area SSP 1207	369308 Norm INT*Area SSP 1208	371707 Norm INT*Area SSP 1210	244303 Norm INT*Area SSP 1401	159054 Norm INT*Area SSP 1402	136152 Norm INT*Area SSP 1403	79607 Norm INT*Area SSP 1501	141614 Norm INT*Area SSP 2301
48509 Norm INT*Area SSP 2302	115673 Norm INT*Area SSP 2303	59729 Norm INT*Area SSP 2304	246819 Norm INT*Area SSP 2305	173948 Norm INT*Area SSP 2401	163297 Norm INT*Area SSP 2402	267526 Norm INT*Area SSP 2403	147986 Norm INT*Area SSP 2501	91460 Norm INT*Area SSP 2601	147798 Norm INT*Area SSP 2602	110602 Norm INT*Area SSP 2603
150518 Norm INT*Area SSP 2604	149708 Norm INT*Area SSP 3301	428299 Norm INT*Area SSP 3302	105552 Norm INT*Area SSP 3401	397242 Norm INT*Area SSP 3402	34328 Norm INT*Area SSP 3403	105183 Norm INT*Area SSP 3404	136509 Norm INT*Area SSP 3405	90847 Norm INT*Area SSP 3406	53223 Norm INT*Area SSP 3501	195057 Norm INT*Area SSP 3502
173511 Norm INT*Area SSP 3503	124801 Norm INT*Area SSP 3504	218149 Norm INT*Area SSP 3505	181825 Norm INT*Area SSP 3506	156808 Norm INT*Area SSP 3507	513560 Norm INT*Area SSP 3508	66607 Norm INT*Area SSP 3601	101863 Norm INT*Area SSP 3602	109392 Norm INT*Area SSP 3603	123028 Norm INT*Area SSP 4301	452959 Norm INT*Area SSP 4302



Spot Review Tool - SST 347 +SA1 D1

○ All spots
● Analysis set: SST 347 +SA1 DAY 1 (72 spots)
Sort by ● SSP ○ DSN ○ Trend ○ Quantity
Page number: 2 of 2

405406 Norm INT*Area SSP 4303	346034 Norm INT*Area SSP 4304	181999 Norm INT*Area SSP 4305	143874 Norm INT*Area SSP 4401	141554 Norm INT*Area SSP 4402	201005 Norm INT*Area SSP 4403	128807 Norm INT*Area SSP 4404	114627 Norm INT*Area SSP 4405	136194 Norm INT*Area SSP 4406	253056 Norm INT*Area SSP 4407	137104 Norm INT*Area SSP 4408
180071 Norm INT*Area SSP 4409	333363 Norm INT*Area SSP 4501	224835 Norm INT*Area SSP 4502	121446 Norm INT*Area SSP 4503	301503 Norm INT*Area SSP 4504	118099 Norm INT*Area SSP 4601	119675 Norm INT*Area SSP 5201	1088727 Norm INT*Area SSP 5301	734983 Norm INT*Area SSP 5501	532446 Norm INT*Area SSP 5502	1102875 Norm INT*Area SSP 5503
747367 Norm INT*Area SSP 5504	116452 Norm INT*Area SSP 5505	123423 Norm INT*Area SSP 5506	595374 Norm INT*Area SSP 5603	111374 Norm INT*Area SSP 6301	316102 Norm INT*Area SSP 7301					

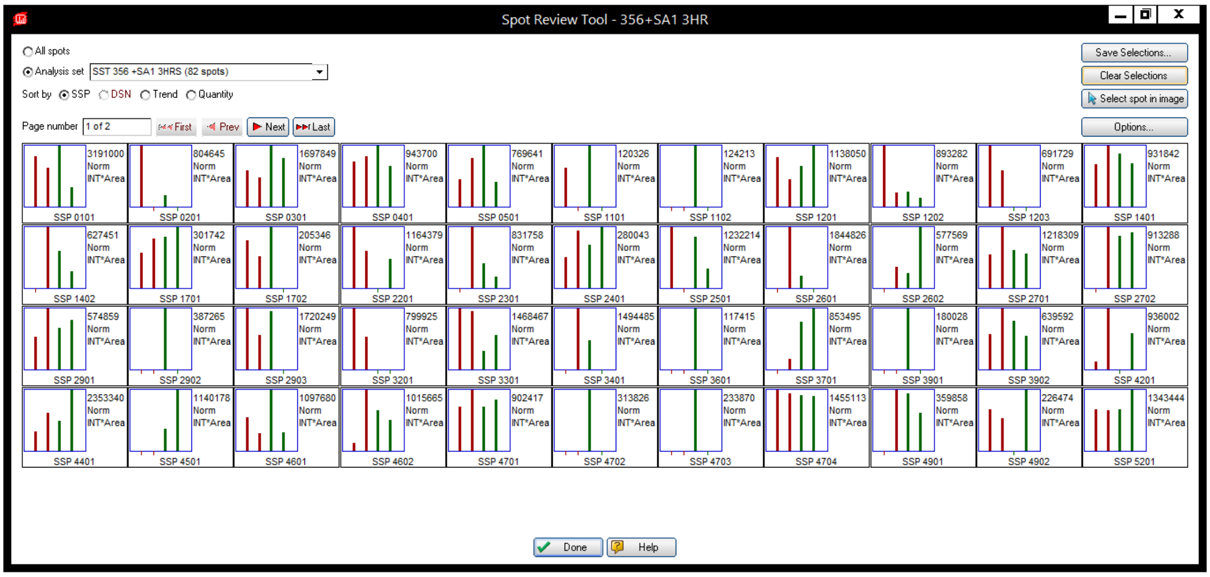


○ All spots
● Analysis set SST356 + SA1 DAY 3 (82 spots)
Sort by ● SSP ○ DSN ○ Trend ○ Quantity
Page number 2 of 2

1884781 Norm INT*Area SSP 5801	1351336 Norm INT*Area SSP 5802	351563 Norm INT*Area SSP 5901	8230249 Norm INT*Area SSP 6201	672159 Norm INT*Area SSP 6301	566197 Norm INT*Area SSP 6302	377028 Norm INT*Area SSP 6401	1258191 Norm INT*Area SSP 6402	575946 Norm INT*Area SSP 6403	475816 Norm INT*Area SSP 6601	394319 Norm INT*Area SSP 6701
3017997 Norm INT*Area SSP 6702	2696465 Norm INT*Area SSP 6801	692870 Norm INT*Area SSP 7201	4153872 Norm INT*Area SSP 7202	677115 Norm INT*Area SSP 7203	2427890 Norm INT*Area SSP 7401	1276972 Norm INT*Area SSP 7501	321419 Norm INT*Area SSP 7502	1098737 Norm INT*Area SSP 7601	740050 Norm INT*Area SSP 7701	2859319 Norm INT*Area SSP 7702
743423 Norm INT*Area SSP 7703	4834472 Norm INT*Area SSP 7801	9656171 Norm INT*Area SSP 7802	326390 Norm INT*Area SSP 8305	7385830 Norm INT*Area SSP 8401	846585 Norm INT*Area SSP 8601	1215955 Norm INT*Area SSP 8602	2502591 Norm INT*Area SSP 8701	2936534 Norm INT*Area SSP 8801	1623574 Norm INT*Area SSP 8802	1785571 Norm INT*Area SSP 8803
96785 Norm INT*Area SSP 9101	620218 Norm INT*Area SSP 9201	1276500 Norm INT*Area SSP 9202	794697 Norm INT*Area SSP 9301	1210776 Norm INT*Area SSP 9302						

○ All spots
● Analysis set SST 356 +SA1 3HRS (82 spots)
Sort by ● SSP ○ DSN ○ Trend ○ Quantity
Page number 1 of 2

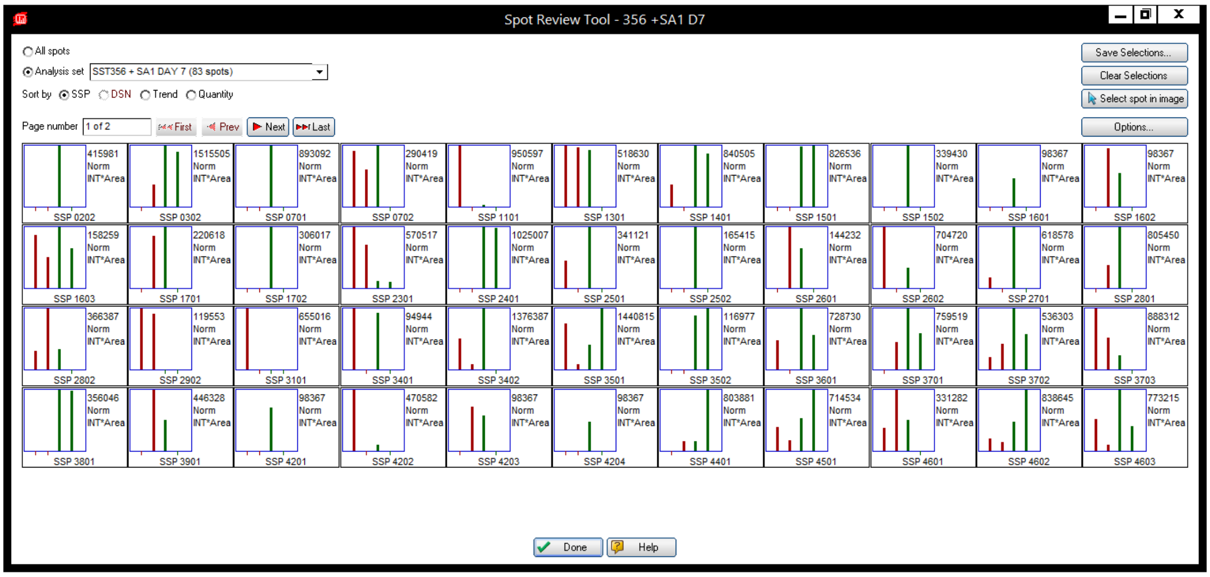
3191000 Norm INT*Area SSP 0101	804645 Norm INT*Area SSP 0201	1697849 Norm INT*Area SSP 0301	943700 Norm INT*Area SSP 0401	769641 Norm INT*Area SSP 0501	120326 Norm INT*Area SSP 1101	124213 Norm INT*Area SSP 1102	1138050 Norm INT*Area SSP 1201	893282 Norm INT*Area SSP 1202	691729 Norm INT*Area SSP 1203	931842 Norm INT*Area SSP 1401
627451 Norm INT*Area SSP 1402	301742 Norm INT*Area SSP 1701	205346 Norm INT*Area SSP 1702	1164379 Norm INT*Area SSP 2201	831758 Norm INT*Area SSP 2301	280043 Norm INT*Area SSP 2401	1232214 Norm INT*Area SSP 2501	1844826 Norm INT*Area SSP 2601	577569 Norm INT*Area SSP 2602	1218309 Norm INT*Area SSP 2701	913288 Norm INT*Area SSP 2702
574859 Norm INT*Area SSP 2901	387265 Norm INT*Area SSP 2902	1720249 Norm INT*Area SSP 2903	799925 Norm INT*Area SSP 3201	1468467 Norm INT*Area SSP 3301	1494485 Norm INT*Area SSP 3401	117415 Norm INT*Area SSP 3601	853495 Norm INT*Area SSP 3701	180028 Norm INT*Area SSP 3901	639592 Norm INT*Area SSP 3902	936002 Norm INT*Area SSP 4201
2353340 Norm INT*Area SSP 4401	1140178 Norm INT*Area SSP 4501	1097680 Norm INT*Area SSP 4601	1015665 Norm INT*Area SSP 4602	902417 Norm INT*Area SSP 4701	313826 Norm INT*Area SSP 4702	233870 Norm INT*Area SSP 4703	1455113 Norm INT*Area SSP 4704	359858 Norm INT*Area SSP 4901	226474 Norm INT*Area SSP 4902	1343444 Norm INT*Area SSP 5201



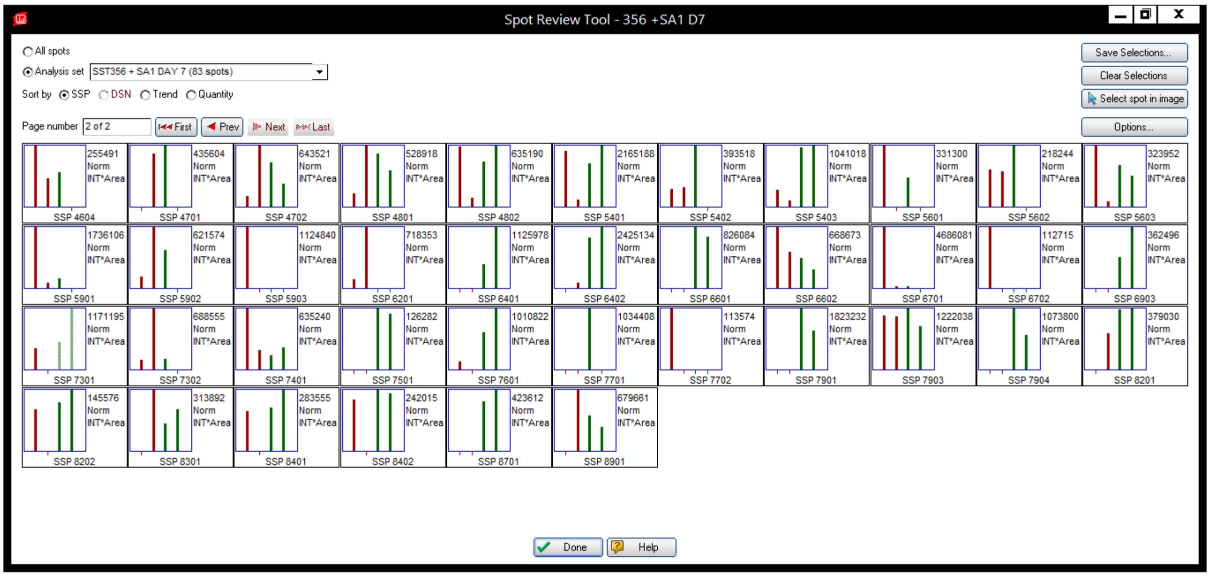
Spot Review Tool - 356+SA1 3HR

○ All spots
● Analysis set SST 356 +SA1 3HRS (82 spots)
Sort by ● SSP ○ DSN ○ Trend ○ Quantity
Page number 1 of 2

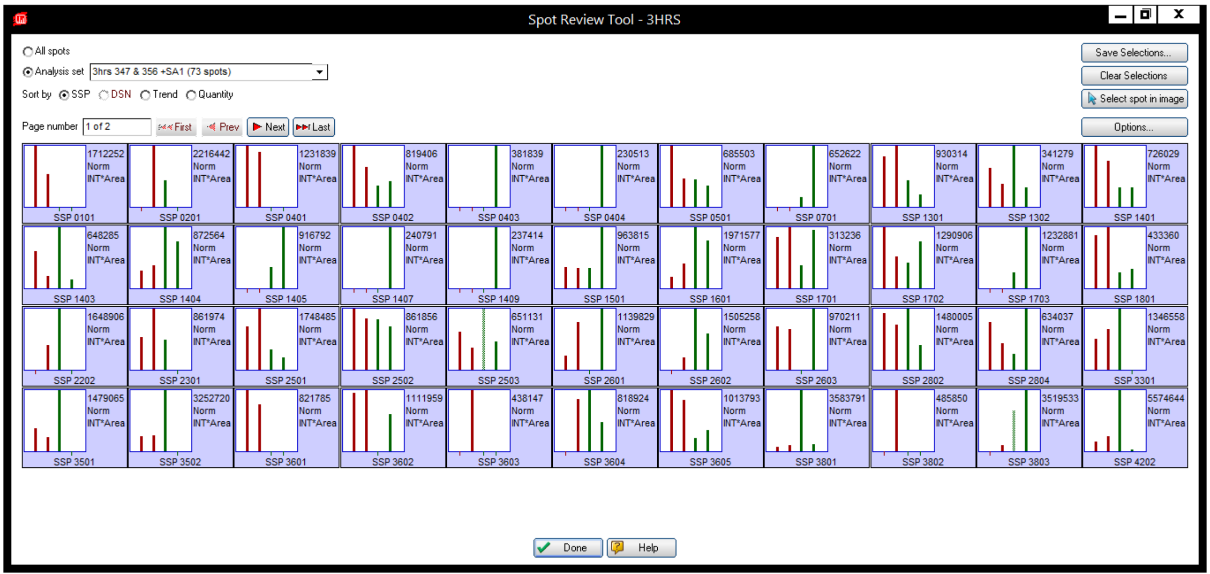
3191000 Norm INT*Area SSP 0101	804645 Norm INT*Area SSP 0201	1697849 Norm INT*Area SSP 0301	943700 Norm INT*Area SSP 0401	769641 Norm INT*Area SSP 0501	120326 Norm INT*Area SSP 1101	124213 Norm INT*Area SSP 1102	1138050 Norm INT*Area SSP 1201	893282 Norm INT*Area SSP 1202	691729 Norm INT*Area SSP 1203	931842 Norm INT*Area SSP 1401
627451 Norm INT*Area SSP 1402	301742 Norm INT*Area SSP 1701	205346 Norm INT*Area SSP 1702	1164379 Norm INT*Area SSP 2201	831758 Norm INT*Area SSP 2301	280043 Norm INT*Area SSP 2401	1232214 Norm INT*Area SSP 2501	1844826 Norm INT*Area SSP 2601	577569 Norm INT*Area SSP 2602	1218309 Norm INT*Area SSP 2701	913288 Norm INT*Area SSP 2702
574859 Norm INT*Area SSP 2901	387265 Norm INT*Area SSP 2902	1720249 Norm INT*Area SSP 2903	799925 Norm INT*Area SSP 3201	1468467 Norm INT*Area SSP 3301	1494485 Norm INT*Area SSP 3401	117415 Norm INT*Area SSP 3601	853495 Norm INT*Area SSP 3701	180028 Norm INT*Area SSP 3901	639592 Norm INT*Area SSP 3902	936002 Norm INT*Area SSP 4201
2353340 Norm INT*Area SSP 4401	1140178 Norm INT*Area SSP 4501	1097680 Norm INT*Area SSP 4601	1015665 Norm INT*Area SSP 4602	902417 Norm INT*Area SSP 4701	313826 Norm INT*Area SSP 4702	233870 Norm INT*Area SSP 4703	1455113 Norm INT*Area SSP 4704	359858 Norm INT*Area SSP 4901	226474 Norm INT*Area SSP 4902	1343444 Norm INT*Area SSP 5201


Spot Review Tool - 356 +SA1 D7

○ All spots
● Analysis set SST356 + SA1 DAY 7 (83 spots)
Sort by ● SSP ○ DSN ○ Trend ○ Quantity
Page number 1 of 2


Spot Review Tool - 356 +SA1 D7

○ All spots
● Analysis set SST356 + SA1 DAY 7 (83 spots)
Sort by ● SSP ○ DSN ○ Trend ○ Quantity
Page number 2 of 2



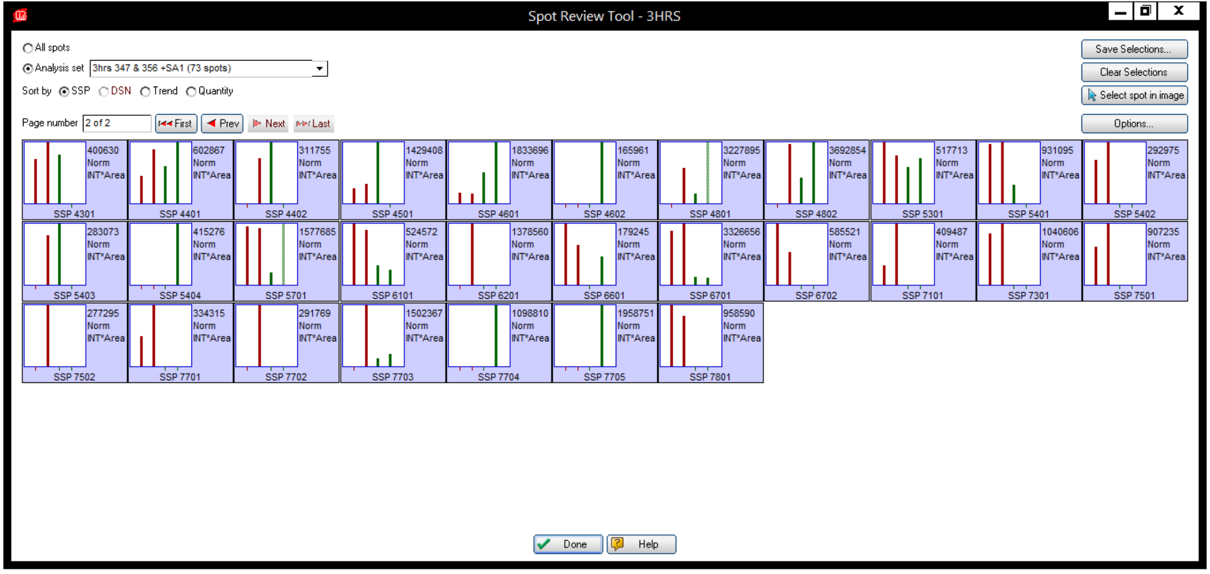
○ All spots

● Analysis set 3hrs 347 & 356 +SA1 (73 spots)

Sort by ● SSP ○ DSN ○ Trend ○ Quantity

Page number 1 of 2

1712252 Norm INT*Area SSP 0101	2216442 Norm INT*Area SSP 0201	1231839 Norm INT*Area SSP 0401	819406 Norm INT*Area SSP 0402	381839 Norm INT*Area SSP 0403	230513 Norm INT*Area SSP 0404	685503 Norm INT*Area SSP 0501	652622 Norm INT*Area SSP 0701	930314 Norm INT*Area SSP 1301	341279 Norm INT*Area SSP 1302	726029 Norm INT*Area SSP 1401
648285 Norm INT*Area SSP 1403	872564 Norm INT*Area SSP 1404	916792 Norm INT*Area SSP 1405	240791 Norm INT*Area SSP 1407	237414 Norm INT*Area SSP 1409	963815 Norm INT*Area SSP 1501	1971577 Norm INT*Area SSP 1601	313236 Norm INT*Area SSP 1701	1290906 Norm INT*Area SSP 1702	1232881 Norm INT*Area SSP 1703	433360 Norm INT*Area SSP 1801
1648906 Norm INT*Area SSP 2202	861974 Norm INT*Area SSP 2301	1748485 Norm INT*Area SSP 2501	861856 Norm INT*Area SSP 2502	651131 Norm INT*Area SSP 2503	1139829 Norm INT*Area SSP 2601	1505258 Norm INT*Area SSP 2602	970211 Norm INT*Area SSP 2603	1480005 Norm INT*Area SSP 2802	634037 Norm INT*Area SSP 2804	1346558 Norm INT*Area SSP 3301
1479065 Norm INT*Area SSP 3501	3252720 Norm INT*Area SSP 3502	821785 Norm INT*Area SSP 3601	1111959 Norm INT*Area SSP 3602	438147 Norm INT*Area SSP 3603	818924 Norm INT*Area SSP 3604	1013793 Norm INT*Area SSP 3605	3583791 Norm INT*Area SSP 3801	485850 Norm INT*Area SSP 3802	3519533 Norm INT*Area SSP 3803	5574644 Norm INT*Area SSP 4202



○ All spots

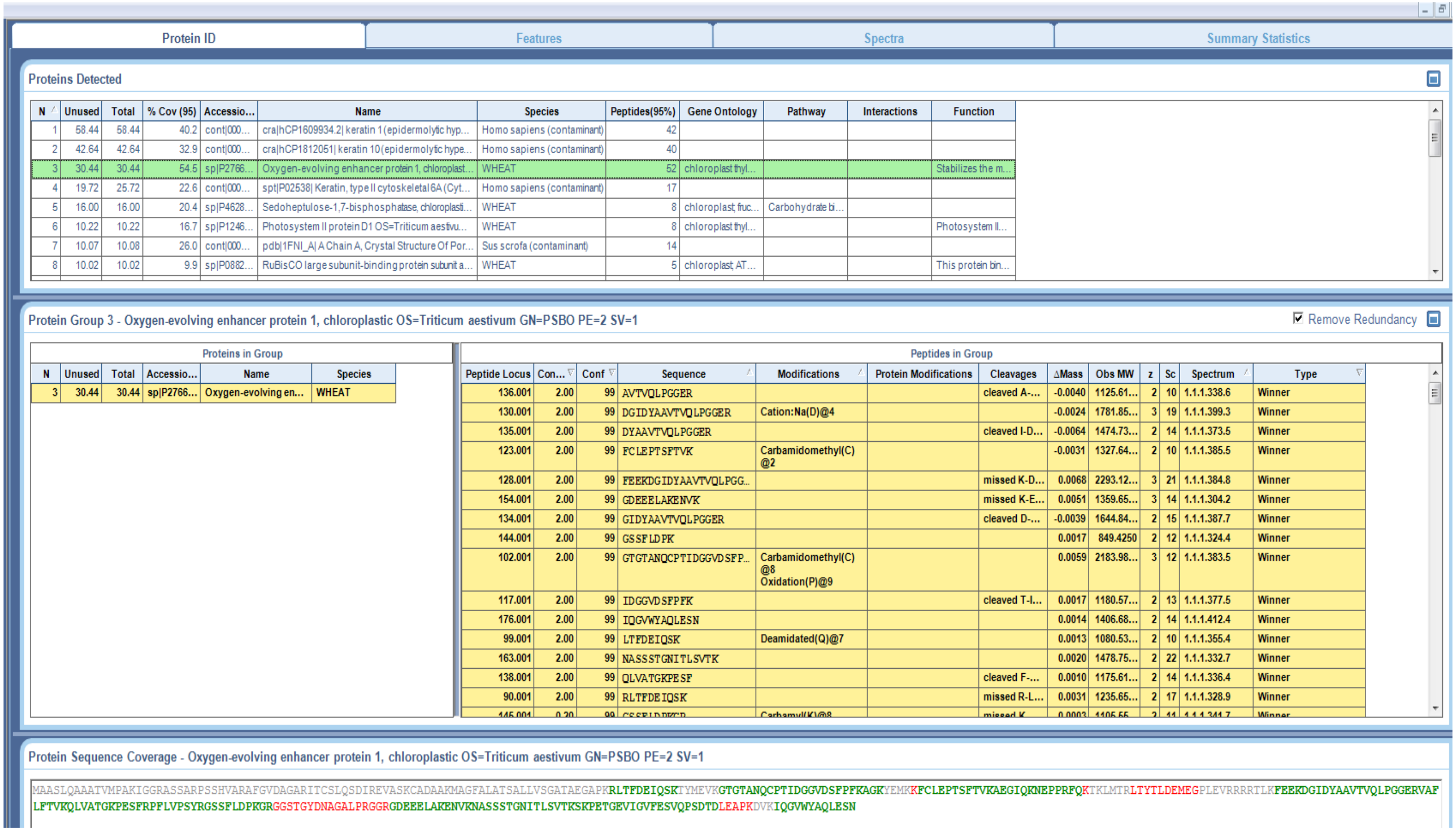
● Analysis set 3hrs 347 & 356 +SA1 (73 spots)

Sort by ● SSP ○ DSN ○ Trend ○ Quantity

Page number 2 of 2

400630 Norm INT*Area SSP 4301	602867 Norm INT*Area SSP 4401	311755 Norm INT*Area SSP 4402	1429408 Norm INT*Area SSP 4501	1833696 Norm INT*Area SSP 4601	165961 Norm INT*Area SSP 4602	3227895 Norm INT*Area SSP 4801	3692854 Norm INT*Area SSP 4802	517713 Norm INT*Area SSP 5301	931095 Norm INT*Area SSP 5401	292975 Norm INT*Area SSP 5402
283073 Norm INT*Area SSP 5403	415276 Norm INT*Area SSP 5404	1577685 Norm INT*Area SSP 5701	524572 Norm INT*Area SSP 6101	1378560 Norm INT*Area SSP 6201	179245 Norm INT*Area SSP 6601	3326656 Norm INT*Area SSP 6701	585521 Norm INT*Area SSP 6702	409487 Norm INT*Area SSP 7101	1040606 Norm INT*Area SSP 7301	907235 Norm INT*Area SSP 7501
277295 Norm INT*Area SSP 7502	334315 Norm INT*Area SSP 7701	291769 Norm INT*Area SSP 7702	1502367 Norm INT*Area SSP 7703	1098810 Norm INT*Area SSP 7704	1958751 Norm INT*Area SSP 7705	958590 Norm INT*Area SSP 7801				

Appendix D: Protein pilots obtained using AB SCIEX Protein Pilot™ Software 5.0



Proteins Detected

N	Unused	Total	% Cov (95)	Accessio...	Name	Species	Peptides(95%)	Gene Ontology	Pathway	Interactions	Function
1	58.44	58.44	40.2	cont|000...	cra|hCP1609934.2| keratin 1 (epidermolytic hyp...	Homo sapiens (contaminant)	42				
2	42.64	42.64	32.9	cont|000...	cra|hCP1812051| keratin 10 (epidermolytic hype...	Homo sapiens (contaminant)	40				
3	30.44	30.44	54.5	sp|P2766...	Oxygen-evolving enhancer protein 1, chloroplast...	WHEAT	52	chloroplast thyl...			Stabilizes the m...
4	19.72	25.72	22.6	cont|000...	spt|P02538| Keratin, type II cytoskeletal 6A (Cyt...	Homo sapiens (contaminant)	17				
5	16.00	16.00	20.4	sp|P4628...	Sedoheptulose-1,7-bisphosphatase, chloroplasti...	WHEAT	8	chloroplast; fruc...	Carbohydrate bi...		
6	10.22	10.22	16.7	sp|P1246...	Photosystem II protein D1 OS=Triticum aestivu...	WHEAT	8	chloroplast thyl...			Photosystem II...
7	10.07	10.08	26.0	cont|000...	pdb|1FNI_A| A Chain A, Crystal Structure Of Por...	Sus scrofa (contaminant)	14				
8	10.02	10.02	9.9	sp|P0882...	RuBisCO large subunit-binding protein subunit a...	WHEAT	5	chloroplast; AT...			This protein bin...

Protein Group 3 - Oxygen-evolving enhancer protein 1, chloroplastic OS=Triticum aestivum GN=PSBO PE=2 SV=1

☑ Remove Redundancy

Proteins in Group

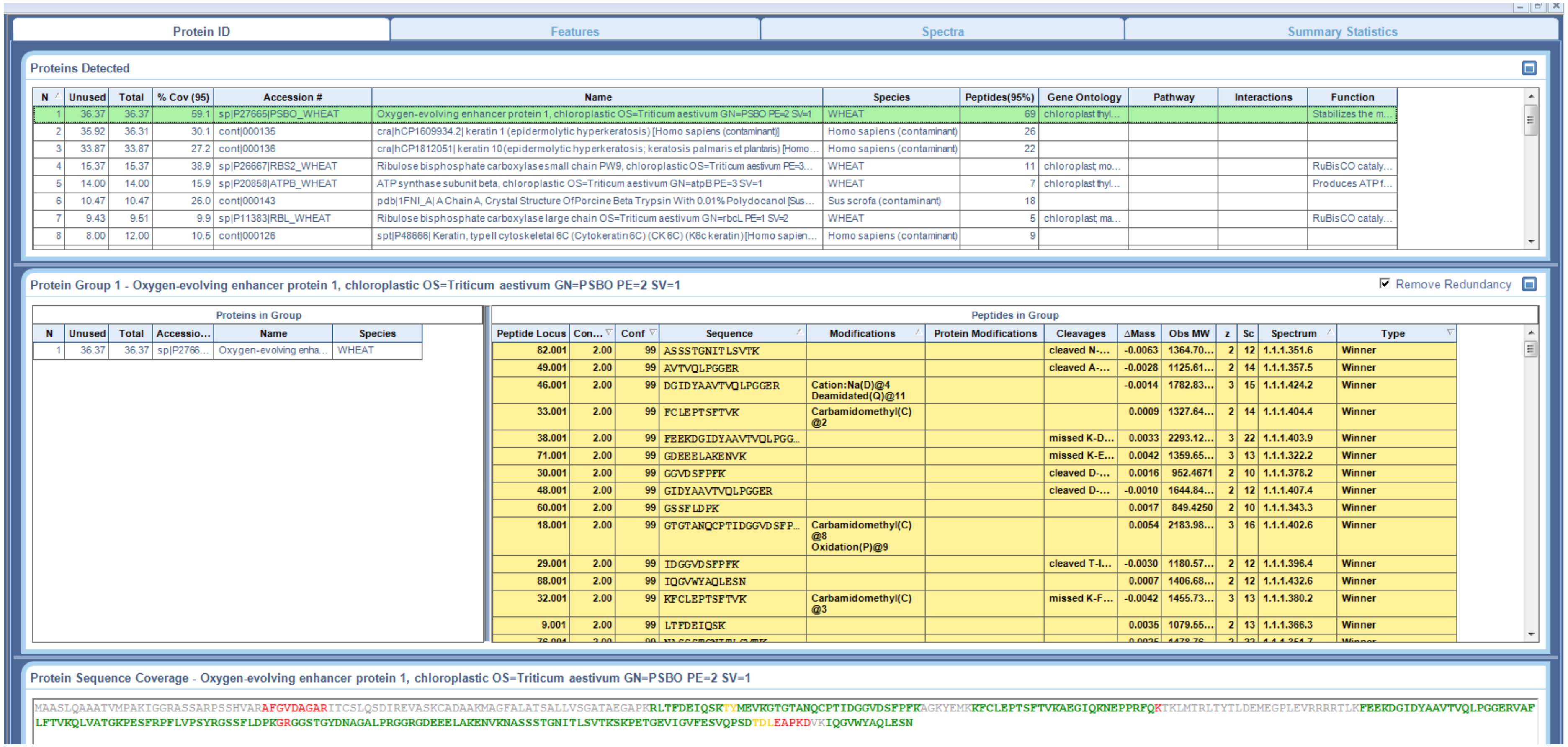
N	Unused	Total	Accessio...	Name	Species
3	30.44	30.44	sp|P2766...	Oxygen-evolving en...	WHEAT

Peptides in Group

Peptide Locus	Con...	Conf	Sequence	Modifications	Protein Modifications	Cleavages	ΔMass	Obs MW	z	Sc	Spectrum	Type
136.001	2.00	99	AVTVQLPGGER			cleaved A-...	-0.0040	1125.61...	2	10	1.1.1.338.6	Winner
130.001	2.00	99	DGIDYAAVTVQLPGGER	Cation:Na(D)@4			-0.0024	1781.85...	3	19	1.1.1.399.3	Winner
135.001	2.00	99	DYAAVTVQLPGGER			cleaved I-D...	-0.0064	1474.73...	2	14	1.1.1.373.5	Winner
123.001	2.00	99	FCLEPTSFTVK	Carbamidomethyl(C)@2			-0.0031	1327.64...	2	10	1.1.1.385.5	Winner
128.001	2.00	99	FEEKDGIDYAAVTVQLPGG...			missed K-D...	0.0068	2293.12...	3	21	1.1.1.384.8	Winner
154.001	2.00	99	GDEEELAKENVK			missed K-E...	0.0051	1359.65...	3	14	1.1.1.304.2	Winner
134.001	2.00	99	GIDYAAVTVQLPGGER			cleaved D-...	-0.0039	1644.84...	2	15	1.1.1.387.7	Winner
144.001	2.00	99	GSSFLDPK				0.0017	849.4250	2	12	1.1.1.324.4	Winner
102.001	2.00	99	GTGTANQCPTIDGGVDSFP...	Carbamidomethyl(C)@8 Oxidation(P)@9			0.0059	2183.98...	3	12	1.1.1.383.5	Winner
117.001	2.00	99	IDGGVDSFPFK			cleaved T-I...	0.0017	1180.57...	2	13	1.1.1.377.5	Winner
176.001	2.00	99	IQGVWYAQLESN				0.0014	1406.68...	2	14	1.1.1.412.4	Winner
99.001	2.00	99	LTFDEIQSK	Deamidated(Q)@7			0.0013	1080.53...	2	10	1.1.1.355.4	Winner
163.001	2.00	99	NASSSTGNITLSVTK				0.0020	1478.75...	2	22	1.1.1.332.7	Winner
138.001	2.00	99	QLVATGKPESF			cleaved F-...	0.0010	1175.61...	2	14	1.1.1.336.4	Winner
90.001	2.00	99	RLTFDEIQSK			missed R-L...	0.0031	1235.65...	2	17	1.1.1.328.9	Winner

Protein Sequence Coverage - Oxygen-evolving enhancer protein 1, chloroplastic OS=Triticum aestivum GN=PSBO PE=2 SV=1

MAASLQAAATVMPAKIGGRASSARPSSHVARAFGVDAGARITCSLQSDIREVASKCADAAKMAGFALATSALLVSGATAEGAPKRLTFDEIQSKTYMEVKGTGTANQCPTIDGGVDSFPFKAGKYEMKKFCLEPTSFTVKAEGIQKNEPPRFQKTKLMTRLTYTLDEMEGPLEVRRRRTLKFEEKDGIDYAAVTVQLPGGERVAF
LFTVKQLVATGKPESFRPFLVPSYRGSSFLDPKGRGGSTGYDNAGALPRGGRGDEEELAKENVKNASSSTGNITLSVTKSKPETGEVIGVFESVQPSDTDLEAPKDVKIQGVWYAQLESN



Proteins Detected

N	Unused	Total	% Cov (95)	Accession #	Name	Species	Peptides(95%)	Gene Ontology	Pathway	Interactions	Function
1	36.37	36.37	59.1	sp|P27665|PSBO_WHEAT	Oxygen-evolving enhancer protein 1, chloroplastic OS=Triticum aestivum GN=PSBO PE=2 SV=1	WHEAT	69	chloroplast thyl...			Stabilizes the m...
2	35.92	36.31	30.1	cont|000135	cra|hCP1609934.2| keratin 1 (epidermolytic hyperkeratosis) [Homo sapiens (contaminant)]	Homo sapiens (contaminant)	26				
3	33.87	33.87	27.2	cont|000136	cra|hCP1812051| keratin 10 (epidermolytic hyperkeratosis; keratosis palmaris et plantaris) [Homo...	Homo sapiens (contaminant)	22				
4	15.37	15.37	38.9	sp|P26667|RBS2_WHEAT	Ribulose bisphosphate carboxylase small chain PW9, chloroplastic OS=Triticum aestivum PE=3...	WHEAT	11	chloroplast; mo...			RuBisCO cataly...
5	14.00	14.00	15.9	sp|P20858|ATPB_WHEAT	ATP synthase subunit beta, chloroplastic OS=Triticum aestivum GN=atpB PE=3 SV=1	WHEAT	7	chloroplast thyl...			Produces ATP f...
6	10.47	10.47	26.0	cont|000143	pdb|1FNI_A| A Chain A, Crystal Structure Of Porcine Beta Trypsin With 0.01% Polydocanol [Sus...	Sus scrofa (contaminant)	18				
7	9.43	9.51	9.9	sp|P11383|RBL_WHEAT	Ribulose bisphosphate carboxylase large chain OS=Triticum aestivum GN=rbcL PE=1 SV=2	WHEAT	5	chloroplast; ma...			RuBisCO cataly...
8	8.00	12.00	10.5	cont|000126	spt|P48666| Keratin, type II cytoskeletal 6C (Cytokeratin 6C) (CK 6C) (K6c keratin) [Homo sapien...	Homo sapiens (contaminant)	9				

Protein Group 1 - Oxygen-evolving enhancer protein 1, chloroplastic OS=Triticum aestivum GN=PSBO PE=2 SV=1

☑ Remove Redundancy

Proteins in Group

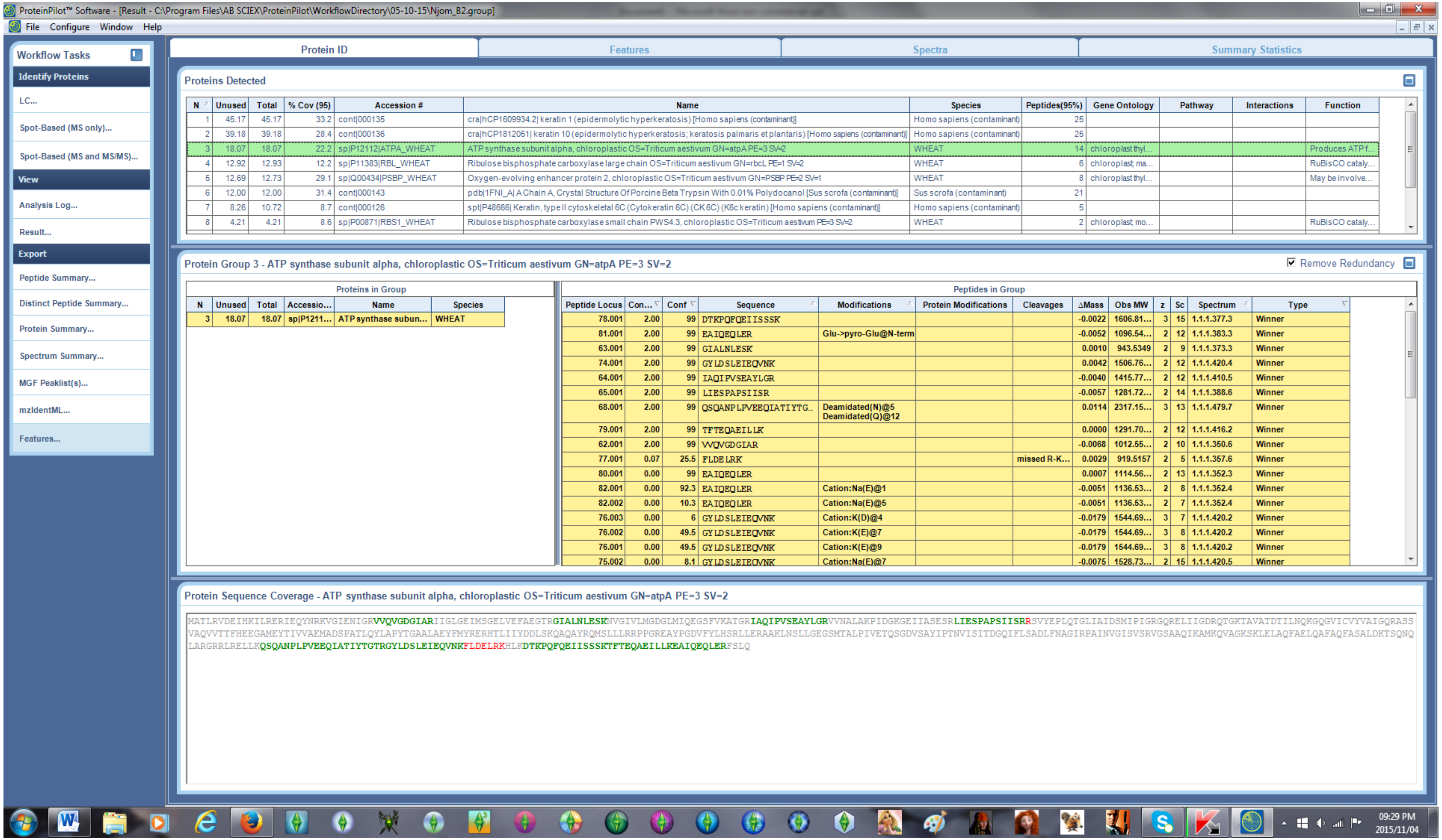
N	Unused	Total	Accessio...	Name	Species
1	36.37	36.37	sp|P2766...	Oxygen-evolving enha...	WHEAT

Peptides in Group

Peptide Locus	Con...	Conf	Sequence	Modifications	Protein Modifications	Cleavages	ΔMass	Obs MW	z	Sc	Spectrum	Type
82.001	2.00	99	ASSSTGNITLSVTK			cleaved N-...	-0.0063	1364.70...	2	12	1.1.1.351.6	Winner
49.001	2.00	99	AVTVQLPGGER			cleaved A-...	-0.0028	1125.61...	2	14	1.1.1.357.5	Winner
46.001	2.00	99	DGIDYAAVTVQLPGGER	Cation:Na(D)@4 Deamidated(Q)@11			-0.0014	1782.83...	3	15	1.1.1.424.2	Winner
33.001	2.00	99	FCLEPTSFTVK	Carbamidomethyl(C)@2			0.0009	1327.64...	2	14	1.1.1.404.4	Winner
38.001	2.00	99	FEEKDGIDYAAVTVQLPGG...			missed K-D...	0.0033	2293.12...	3	22	1.1.1.403.9	Winner
71.001	2.00	99	GDEEELAKENVK			missed K-E...	0.0042	1359.65...	3	13	1.1.1.322.2	Winner
30.001	2.00	99	GGVDSFPFK			cleaved D-...	0.0016	952.4671	2	10	1.1.1.378.2	Winner
48.001	2.00	99	GIDYAAVTVQLPGGER			cleaved D-...	-0.0010	1644.84...	2	12	1.1.1.407.4	Winner
60.001	2.00	99	GSSFLDPK				0.0017	849.4250	2	10	1.1.1.343.3	Winner
18.001	2.00	99	GTGTANQCPTIDGGVDSFP...	Carbamidomethyl(C)@8 Oxidation(P)@9			0.0054	2183.98...	3	16	1.1.1.402.6	Winner
29.001	2.00	99	IDGGVDSFPFK			cleaved T-I...	-0.0030	1180.57...	2	12	1.1.1.396.4	Winner
88.001	2.00	99	IQGVWYAQLESN				0.0007	1406.68...	2	12	1.1.1.432.6	Winner
32.001	2.00	99	KFCLEPTSFTVK	Carbamidomethyl(C)@3		missed K-F...	-0.0042	1455.73...	3	13	1.1.1.380.2	Winner
9.001	2.00	99	LTFDEIQSK				0.0035	1079.55...	2	13	1.1.1.366.3	Winner

Protein Sequence Coverage - Oxygen-evolving enhancer protein 1, chloroplastic OS=Triticum aestivum GN=PSBO PE=2 SV=1

MAASLQAAATVMPAKIGGRASSARPSSHVARAFGVDAGARITCSLQSDIREVASKCADAAKMAGFALATSALLVSGATAEGAPKRLTFDEIQSKTYMEVKGTGTANQCPTIDGGVDSFPFKAGKYEMKKFCLEPTSFTVKAEGIQKNEPPRFQKTKLMTRLTYTLDEMEGPLEVRRRRTLKFEEKDGIDYAAVTVQLPGGERVAF
LFTVKQLVATGKPESFRPFLVPSYRGSSFLDPKGRGGSTGYDNAGALPRGGRGDEEELAKENVKNASSSTGNITLSVTKSKPETGEVIGVFESVQPSDTDLEAPKDVKIQGVWYAQLESN



Proteins Detected

N	Unused	Total	% Cov (95)	Accession #	Name	Species	Peptides(95%)	Gene Ontology	Pathway	Interactions	Function
1	45.17	45.17	33.2	cont|000135	cra|hCP1609934.2| keratin 1 (epidermolytic hyperkeratosis) [Homo sapiens (contaminant)]	Homo sapiens (contaminant)	25				
2	39.18	39.18	28.4	cont|000136	cra|hCP1812051| keratin 10 (epidermolytic hyperkeratosis; keratosis palmaris et plantaris) [Homo sapiens (contaminant)]	Homo sapiens (contaminant)	25				
3	18.07	18.07	22.2	sp|P12112|ATPA_WHEAT	ATP synthase subunit alpha, chloroplastic OS=Triticum aestivum GN=atpA PE=3 SV=2	WHEAT	14	chloroplast thyl...			Produces ATP f...
4	12.92	12.93	12.2	sp|P11383|RBL_WHEAT	Ribulose bisphosphate carboxylase large chain OS=Triticum aestivum GN=rbcL PE=1 SV=2	WHEAT	6	chloroplast; ma...			RuBisCO cataly...
5	12.69	12.73	29.1	sp|Q00434|PSBP_WHEAT	Oxygen-evolving enhancer protein 2, chloroplastic OS=Triticum aestivum GN=PSBP PE=2 SV=1	WHEAT	8	chloroplast thyl...			May be involve...
6	12.00	12.00	31.4	cont|000143	pdb|1FNI_A| A Chain A, Crystal Structure Of Porcine Beta Trypsin With 0.01% Polydocanol [Sus scrofa (contaminant)]	Sus scrofa (contaminant)	21				
7	8.26	10.72	8.7	cont|000126	spt|P48666| Keratin, type II cytoskeletal 6C (Cytokeratin 6C) (CK 6C) (K6c keratin) [Homo sapiens (contaminant)]	Homo sapiens (contaminant)	5				
8	4.21	4.21	8.6	sp|P00871|RBS1_WHEAT	Ribulose bisphosphate carboxylase small chain PWS4.3, chloroplastic OS=Triticum aestivum PE=3 SV=2	WHEAT	2	chloroplast; mo...			RuBisCO cataly...

Protein Group 3 - ATP synthase subunit alpha, chloroplastic OS=Triticum aestivum GN=atpA PE=3 SV=2

☑ Remove Redundancy

Proteins in Group

N	Unused	Total	Accessio...	Name	Species
3	18.07	18.07	sp|P1211...	ATP synthase subun...	WHEAT

Peptides in Group

Peptide Locus	Con...	Conf	Sequence	Modifications	Protein Modifications	Cleavages	ΔMass	Obs MW	z	Sc	Spectrum	Type
78.001	2.00	99	DTKPQFQEIISSSK				-0.0022	1606.81...	3	15	1.1.1.377.3	Winner
81.001	2.00	99	EAIQEQLER	Glu->pyro-Glu@N-term			-0.0052	1096.54...	2	12	1.1.1.383.3	Winner
63.001	2.00	99	GIALNLESK				0.0010	943.5349	2	9	1.1.1.373.3	Winner
74.001	2.00	99	GYLDSLEIEQVNK				0.0042	1506.76...	2	12	1.1.1.420.4	Winner
64.001	2.00	99	IAQIPVSEAYLGR				-0.0040	1415.77...	2	12	1.1.1.410.5	Winner
65.001	2.00	99	LIESPAPSIISR				-0.0057	1281.72...	2	14	1.1.1.388.6	Winner
68.001	2.00	99	QSQANPLPVEEQIATIYTG...	Deamidated(N)@5 Deamidated(Q)@12			0.0114	2317.15...	3	13	1.1.1.479.7	Winner
79.001	2.00	99	TFTEQAEILLK				0.0000	1291.70...	2	12	1.1.1.416.2	Winner
62.001	2.00	99	VVQVGDGIAR				-0.0068	1012.55...	2	10	1.1.1.350.6	Winner
77.001	0.07	25.5	FLDELRK			missed R-K...	0.0029	919.5157	2	5	1.1.1.357.6	Winner
80.001	0.00	99	EAIQEQLER				0.0007	1114.56...	2	13	1.1.1.352.3	Winner
82.001	0.00	92.3	EAIQEQLER	Cation:Na(E)@1			-0.0051	1136.53...	2	8	1.1.1.352.4	Winner
82.002	0.00	10.3	EAIQEQLER	Cation:Na(E)@5			-0.0051	1136.53...	2	7	1.1.1.352.4	Winner
76.003	0.00	6	GYLDSLEIEQVNK	Cation:K(D)@4			-0.0179	1544.69...	3	7	1.1.1.420.2	Winner
76.002	0.00	49.5	GYLDSLEIEQVNK	Cation:K(E)@7			-0.0179	1544.69...	3	8	1.1.1.420.2	Winner
76.001	0.00	49.5	GYLDSLEIEQVNK	Cation:K(E)@9			-0.0179	1544.69...	3	8	1.1.1.420.2	Winner
75.002	0.00	8.1	GYLDSLEIEQVNK	Cation:Na(E)@7			-0.0075	1528.73...	2	15	1.1.1.420.5	Winner

Protein Sequence Coverage - ATP synthase subunit alpha, chloroplastic OS=Triticum aestivum GN=atpA PE=3 SV=2

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MATLRVDEIHKILRERIEQYNRKVGIENIGRVVQVGDGIARIIGLGEIMSGELVEFAEGTRGIALNLESKNVGIVLMGDGLMIQEGSFVKATGRIAQIPVSEAYLGRVVNALAKPIDGKGEIIASESRLIESPAPSIISRRSVYEPLQTGLIAIDSMIPIGRGQRELIIGDRQTGKTAVATDTILNQKGQGVICVYVAIGQRASS
VAQVVTTFHEEGAMEYTIVVAEMADSPATLQYLAPYTGAALAEYFMYRERHTLIIYDDLSKQAQAYRQMSLLLRRPPGREAYPGDVFYLHSRLLERAAKLNSLLGEGSMTALPIVETQSGDVSAYIPTNVISITDGQIFLSADLFNAGIRPAINVGISVSRVGSAAQIKAMKQVAGKSKLELAQFAELQAFAQFASALDKTSQNQ
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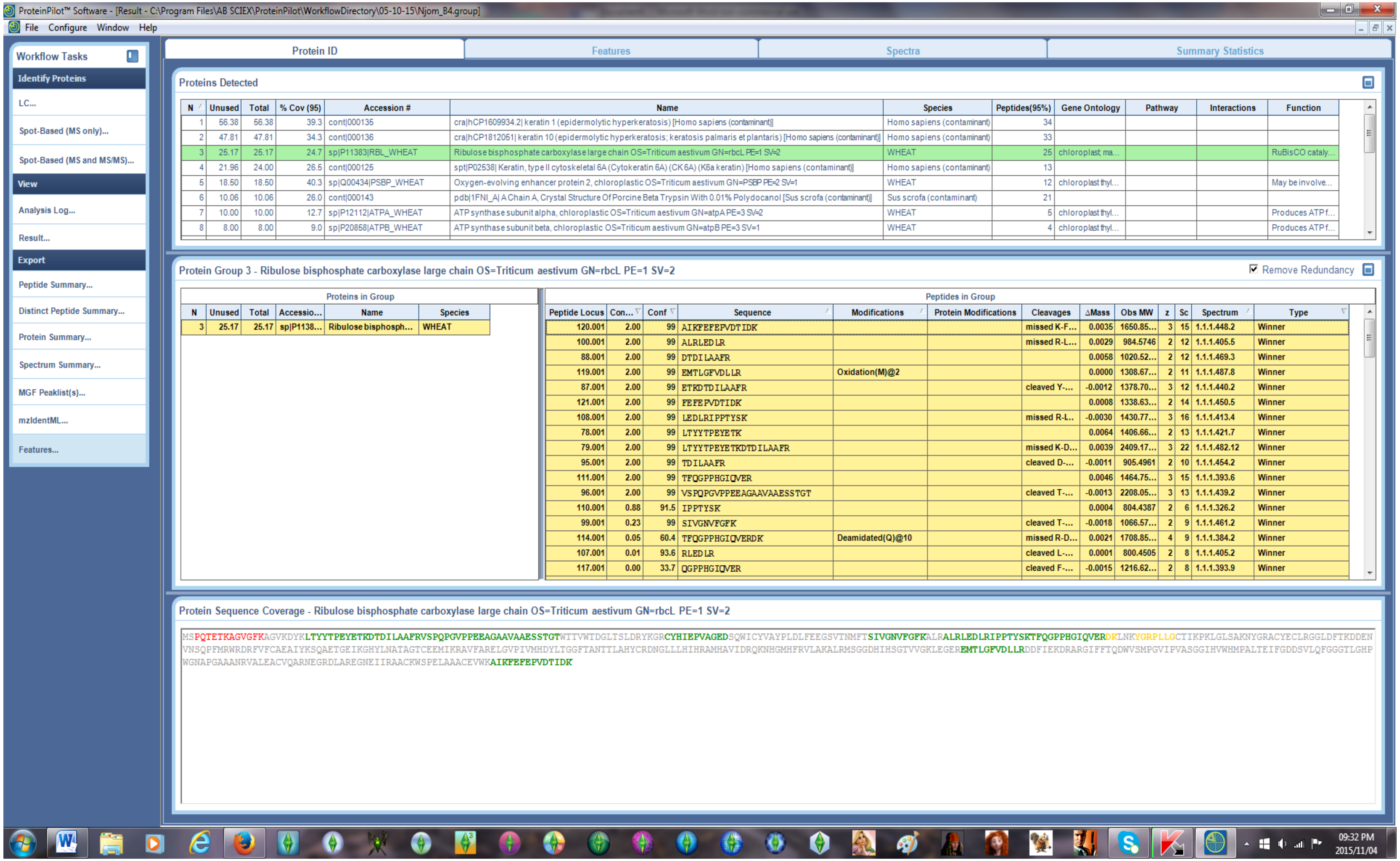

Proteins Detected

N	Unused	Total	% Cov (95)	Accession #	Name	Species	Peptides(95%)	Gene Ontology	Pathway	Interactions	Function
1	56.38	56.38	39.3	cont|000135	cra|hCP1609934.2| keratin 1 (epidermolytic hyperkeratosis) [Homo sapiens (contaminant)]	Homo sapiens (contaminant)	34				
2	47.81	47.81	34.3	cont|000136	cra|hCP1812051| keratin 10 (epidermolytic hyperkeratosis; keratosis palmaris et plantaris) [Homo sapiens (contaminant)]	Homo sapiens (contaminant)	33				
3	25.17	25.17	24.7	sp|P11383|RBL_WHEAT	Ribulose bisphosphate carboxylase large chain OS=Triticum aestivum GN=rbcL PE=1 SV=2	WHEAT	25	chloroplast, ma...			RuBisCO cataly...
4	21.96	24.00	26.5	cont|000125	spt|P02538| Keratin, type II cytoskeletal 6A (Cytokeratin 6A) (CK 6A) (K6a keratin) [Homo sapiens (contaminant)]	Homo sapiens (contaminant)	13				
5	18.50	18.50	40.3	sp|Q00434|PSBP_WHEAT	Oxygen-evolving enhancer protein 2, chloroplastic OS=Triticum aestivum GN=PSBP PE=2 SV=1	WHEAT	12	chloroplast thyl...			May be involve...
6	10.06	10.06	26.0	cont|000143	pdb|1FNI_A| A Chain A, Crystal Structure Of Porcine Beta Trypsin With 0.01% Polydocanol [Sus scrofa (contaminant)]	Sus scrofa (contaminant)	21				
7	10.00	10.00	12.7	sp|P12112|ATPA_WHEAT	ATP synthase subunit alpha, chloroplastic OS=Triticum aestivum GN=atpA PE=3 SV=2	WHEAT	5	chloroplast thyl...			Produces ATP f...
8	8.00	8.00	9.0	sp|P20858|ATPB_WHEAT	ATP synthase subunit beta, chloroplastic OS=Triticum aestivum GN=atpB PE=3 SV=1	WHEAT	4	chloroplast thyl...			Produces ATP f...

Protein Group 3 - Ribulose bisphosphate carboxylase large chain OS=Triticum aestivum GN=rbcL PE=1 SV=2

☑ Remove Redundancy

Proteins in Group

N	Unused	Total	Accessio...	Name	Species
3	25.17	25.17	sp|P1138...	Ribulose bisphosph...	WHEAT

Peptides in Group

Peptide Locus	Con...	Conf	Sequence	Modifications	Protein Modifications	Cleavages	ΔMass	Obs MW	z	Sc	Spectrum	Type
120.001	2.00	99	AIKFEFEPVDTIDK			missed K-F...	0.0035	1650.85...	3	15	1.1.1.448.2	Winner
100.001	2.00	99	ALRLEDLR			missed R-L...	0.0029	984.5746	2	12	1.1.1.405.5	Winner
88.001	2.00	99	DTDILAAFR				0.0058	1020.52...	2	12	1.1.1.469.3	Winner
119.001	2.00	99	EMTLGFVDLLR	Oxidation(M)@2			0.0000	1308.67...	2	11	1.1.1.487.8	Winner
87.001	2.00	99	ETKDTDILAAFR			cleaved Y-...	-0.0012	1378.70...	3	12	1.1.1.440.2	Winner
121.001	2.00	99	FEFEPVDTIDK				0.0008	1338.63...	2	14	1.1.1.450.5	Winner
108.001	2.00	99	LEDLRIPPTYSK			missed R-L...	-0.0030	1430.77...	3	16	1.1.1.413.4	Winner
78.001	2.00	99	LTYYTPEYETK				0.0064	1406.66...	2	13	1.1.1.421.7	Winner
79.001	2.00	99	LTYYTPEYETKDTDILAAFR			missed K-D...	0.0039	2409.17...	3	22	1.1.1.482.12	Winner
95.001	2.00	99	TDILAAFR			cleaved D-...	-0.0011	905.4961	2	10	1.1.1.454.2	Winner
111.001	2.00	99	TFQGPPHGIQVER				0.0046	1464.75...	3	15	1.1.1.393.6	Winner
96.001	2.00	99	VSPQPGVPPEEAGAAVAAESSTGT			cleaved T-...	-0.0013	2208.05...	3	13	1.1.1.439.2	Winner
110.001	0.88	91.5	IPPTYSK				0.0004	804.4387	2	6	1.1.1.326.2	Winner
99.001	0.23	99	SIVGNVFGFK			cleaved T-...	-0.0018	1066.57...	2	9	1.1.1.461.2	Winner
114.001	0.05	60.4	TFQGPPHGIQVERDK	Deamidated(Q)@10		missed R-D...	0.0021	1708.85...	4	9	1.1.1.384.2	Winner
107.001	0.01	93.6	RLEDLR			cleaved L-...	0.0001	800.4505	2	8	1.1.1.405.2	Winner
117.001	0.00	33.7	QGPPHGIQVER			cleaved F-...	-0.0015	1216.62...	2	8	1.1.1.393.9	Winner

Protein Sequence Coverage - Ribulose bisphosphate carboxylase large chain OS=Triticum aestivum GN=rbcL PE=1 SV=2

```
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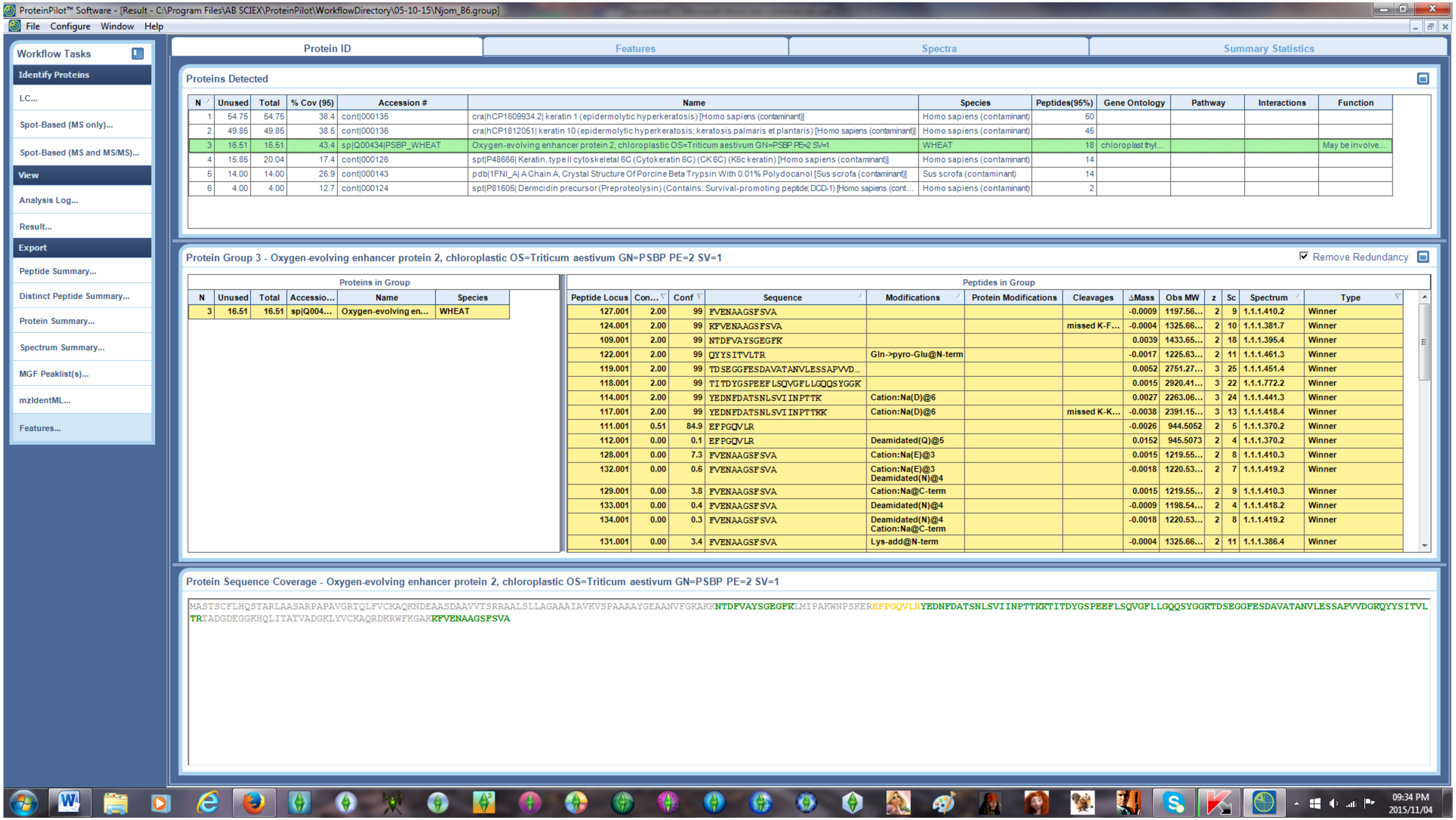

Proteins Detected

N	Unused	Total	% Cov (95)	Accession #	Name	Species	Peptides(95%)	Gene Ontology	Pathway	Interactions	Function
1	54.75	54.75	38.4	cont|000135	cra|hCP1609934.2| keratin 1 (epidermolytic hyperkeratosis) [Homo sapiens (contaminant)]	Homo sapiens (contaminant)	50				
2	49.85	49.85	38.5	cont|000136	cra|hCP1812051| keratin 10 (epidermolytic hyperkeratosis; keratosis palmaris et plantaris) [Homo sapiens (contaminant)]	Homo sapiens (contaminant)	45				
3	16.51	16.51	43.4	sp|Q00434|PSBP_WHEAT	Oxygen-evolving enhancer protein 2, chloroplastic OS=Triticum aestivum GN=PSBP PE=2 SV=1	WHEAT	18	chloroplast thyl...			May be involve...
4	15.85	20.04	17.4	cont|000126	spt|P48666| Keratin, type II cytoskeletal 6C (Cytokeratin 6C) (CK 6C) (K6c keratin) [Homo sapiens (contaminant)]	Homo sapiens (contaminant)	14				
5	14.00	14.00	26.9	cont|000143	pdb|1FNI_A| A Chain A, Crystal Structure Of Porcine Beta Trypsin With 0.01% Polydocanol [Sus scrofa (contaminant)]	Sus scrofa (contaminant)	14				
6	4.00	4.00	12.7	cont|000124	spt|P81605| Dermcidin precursor (Preproteolysin) (Contains: Survival-promoting peptide; DCD-1) [Homo sapiens (cont...	Homo sapiens (contaminant)	2				

Protein Group 3 - Oxygen-evolving enhancer protein 2, chloroplastic OS=Triticum aestivum GN=PSBP PE=2 SV=1

☑ Remove Redundancy

Proteins in Group

N	Unused	Total	Accessio...	Name	Species
3	16.51	16.51	sp|Q004...	Oxygen-evolving en...	WHEAT

Peptides in Group

Peptide Locus	Con...	Conf	Sequence	Modifications	Protein Modifications	Cleavages	ΔMass	Obs MW	z	Sc	Spectrum	Type
127.001	2.00	99	FVENAAGSFSVA				-0.0009	1197.56...	2	9	1.1.1.410.2	Winner
124.001	2.00	99	KFVENAAGSFSVA			missed K-F...	-0.0004	1325.66...	2	10	1.1.1.381.7	Winner
109.001	2.00	99	NTDFVAYSGEGFK				0.0039	1433.65...	2	18	1.1.1.395.4	Winner
122.001	2.00	99	QYYSITVLTR	Gln->pyro-Glu@N-term			-0.0017	1225.63...	2	11	1.1.1.461.3	Winner
119.001	2.00	99	TDSEGGFESDAVATANVLESSAPVVD...				0.0052	2751.27...	3	25	1.1.1.451.4	Winner
118.001	2.00	99	TITDYGSPEEFLSQVGFLLGQQSYGGK				0.0015	2920.41...	3	22	1.1.1.772.2	Winner
114.001	2.00	99	YEDNFDATSNLSVIINPTTK	Cation:Na(D)@6			0.0027	2263.06...	3	24	1.1.1.441.3	Winner
117.001	2.00	99	YEDNFDATSNLSVIINPTTKK	Cation:Na(D)@6		missed K-K...	-0.0038	2391.15...	3	13	1.1.1.418.4	Winner
111.001	0.51	84.9	EFPGQVLR				-0.0026	944.5052	2	5	1.1.1.370.2	Winner
112.001	0.00	0.1	EFPGQVLR	Deamidated(Q)@5			0.0152	945.5073	2	4	1.1.1.370.2	Winner
128.001	0.00	7.3	FVENAAGSFSVA	Cation:Na(E)@3			0.0015	1219.55...	2	8	1.1.1.410.3	Winner
132.001	0.00	0.6	FVENAAGSFSVA	Cation:Na(E)@3 Deamidated(N)@4			-0.0018	1220.53...	2	7	1.1.1.419.2	Winner
129.001	0.00	3.8	FVENAAGSFSVA	Cation:Na@C-term			0.0015	1219.55...	2	9	1.1.1.410.3	Winner
133.001	0.00	0.4	FVENAAGSFSVA	Deamidated(N)@4			-0.0009	1198.54...	2	4	1.1.1.418.2	Winner
134.001	0.00	0.3	FVENAAGSFSVA	Deamidated(N)@4 Cation:Na@C-term			-0.0018	1220.53...	2	8	1.1.1.419.2	Winner
131.001	0.00	3.4	FVENAAGSFSVA	Lys-add@N-term			-0.0004	1325.66...	2	11	1.1.1.386.4	Winner

Protein Sequence Coverage - Oxygen-evolving enhancer protein 2, chloroplastic OS=Triticum aestivum GN=PSBP PE=2 SV=1

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MASTSCFLHQSTARLAASARPAPAVGRTQLFVCKAQKNDEAASDAAVVTSRRAALSLLAGAAAIAVKVSPAAAAYGEAANVFGKAKKNTDFVAYSGEGFKLMIPAKWNPSKEREFPGQVLRYEDNFDATSNLSVIINPTTKKTITDYGSPEEFLSQVGFLLGQQSYGGKTDSEGGFESDAVATANVLESSAPVVDGKQYYSITVL
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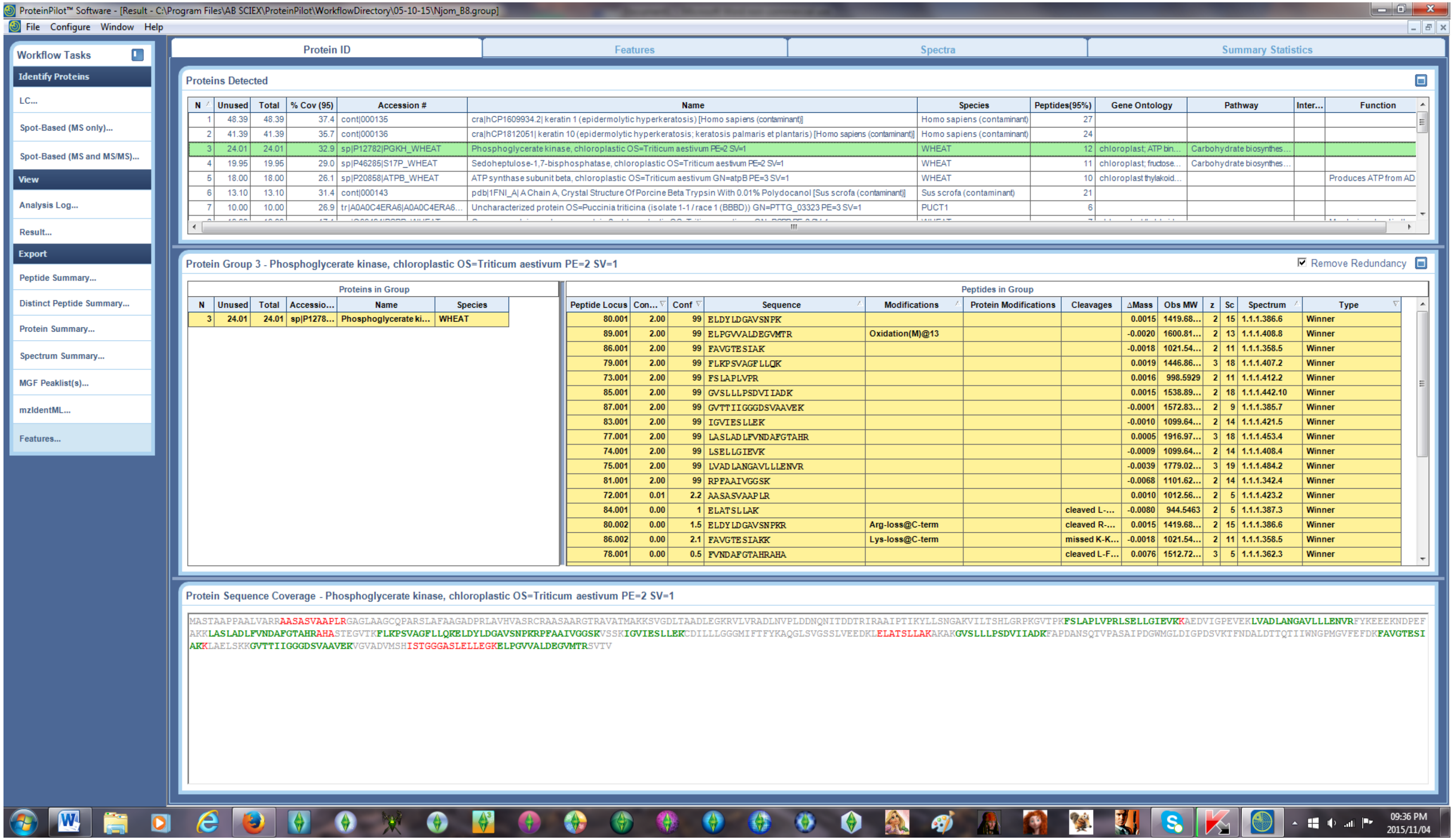

Proteins Detected

N	Unused	Total	% Cov (95)	Accession #	Name	Species	Peptides(95%)	Gene Ontology	Pathway	Inter...	Function
1	48.39	48.39	37.4	cont|000135	cra|hCP1609934.2| keratin 1 (epidermolytic hyperkeratosis) [Homo sapiens (contaminant)]	Homo sapiens (contaminant)	27				
2	41.39	41.39	35.7	cont|000136	cra|hCP1812051| keratin 10 (epidermolytic hyperkeratosis; keratosis palmaris et plantaris) [Homo sapiens (contaminant)]	Homo sapiens (contaminant)	24				
3	24.01	24.01	32.9	sp|P12782|PGKH_WHEAT	Phosphoglycerate kinase, chloroplastic OS=Triticum aestivum PE=2 SV=1	WHEAT	12	chloroplast; ATP bin...	Carbohydrate biosynthes...		
4	19.95	19.95	29.0	sp|P46285|S17P_WHEAT	Sedoheptulose-1,7-bisphosphatase, chloroplastic OS=Triticum aestivum PE=2 SV=1	WHEAT	11	chloroplast; fructose...	Carbohydrate biosynthes...		
5	18.00	18.00	26.1	sp|P20858|ATPB_WHEAT	ATP synthase subunit beta, chloroplastic OS=Triticum aestivum GN=atpB PE=3 SV=1	WHEAT	10	chloroplast thylakoid...			Produces ATP from AD
6	13.10	13.10	31.4	cont|000143	pdb|1FNI_A| A Chain A, Crystal Structure Of Porcine Beta Trypsin With 0.01% Polydocanol [Sus scrofa (contaminant)]	Sus scrofa (contaminant)	21				
7	10.00	10.00	26.9	tr|A0A0C4ERA6|A0A0C4ERA6...	Uncharacterized protein OS=Puccinia triticina (isolate 1-1 / race 1 (BBBD)) GN=PTTG_03323 PE=3 SV=1	PUCT1	6				

Protein Group 3 - Phosphoglycerate kinase, chloroplastic OS=Triticum aestivum PE=2 SV=1

☑ Remove Redundancy

Proteins in Group

N	Unused	Total	Accessio...	Name	Species
3	24.01	24.01	sp|P1278...	Phosphoglycerate ki...	WHEAT

Peptides in Group

Peptide Locus	Con...	Conf	Sequence	Modifications	Protein Modifications	Cleavages	ΔMass	Obs MW	z	Sc	Spectrum	Type
80.001	2.00	99	ELDYLDGAVSNPK				0.0015	1419.68...	2	15	1.1.1.386.6	Winner
89.001	2.00	99	ELPGVVALDEGVMTR	Oxidation(M)@13			-0.0020	1600.81...	2	13	1.1.1.408.8	Winner
86.001	2.00	99	FAVGTESIAK				-0.0018	1021.54...	2	11	1.1.1.358.5	Winner
79.001	2.00	99	FLKPSVAGFLLQK				0.0019	1446.86...	3	18	1.1.1.407.2	Winner
73.001	2.00	99	FSLAPLVPR				0.0016	998.5929	2	11	1.1.1.412.2	Winner
85.001	2.00	99	GVSLLLPSDVIIADK				0.0015	1538.89...	2	18	1.1.1.442.10	Winner
87.001	2.00	99	GVTTIIGGGDSVAAVEK				-0.0001	1572.83...	2	9	1.1.1.385.7	Winner
83.001	2.00	99	IGVIESLLEK				-0.0010	1099.64...	2	14	1.1.1.421.5	Winner
77.001	2.00	99	LASLADLFVNDAFGTAHR				0.0005	1916.97...	3	18	1.1.1.453.4	Winner
74.001	2.00	99	LSELLGIEVK				-0.0009	1099.64...	2	14	1.1.1.408.4	Winner
75.001	2.00	99	LVADLANGAVLLLENVR				-0.0039	1779.02...	3	19	1.1.1.484.2	Winner
81.001	2.00	99	RPFAAIVGGSK				-0.0068	1101.62...	2	14	1.1.1.342.4	Winner
72.001	0.01	2.2	AASASVAAPLR				0.0010	1012.56...	2	5	1.1.1.423.2	Winner
84.001	0.00	1	ELATSLLAK			cleaved L-...	-0.0080	944.5463	2	5	1.1.1.387.3	Winner
80.002	0.00	1.5	ELDYLDGAVSNPKR	Arg-loss@C-term		cleaved R-...	0.0015	1419.68...	2	15	1.1.1.386.6	Winner
86.002	0.00	2.1	FAVGTESIAKK	Lys-loss@C-term		missed K-K...	-0.0018	1021.54...	2	11	1.1.1.358.5	Winner
78.001	0.00	0.5	FVNDAFGTAHRAHA			cleaved L-F...	0.0076	1512.72...	3	5	1.1.1.362.3	Winner

Protein Sequence Coverage - Phosphoglycerate kinase, chloroplastic OS=Triticum aestivum PE=2 SV=1

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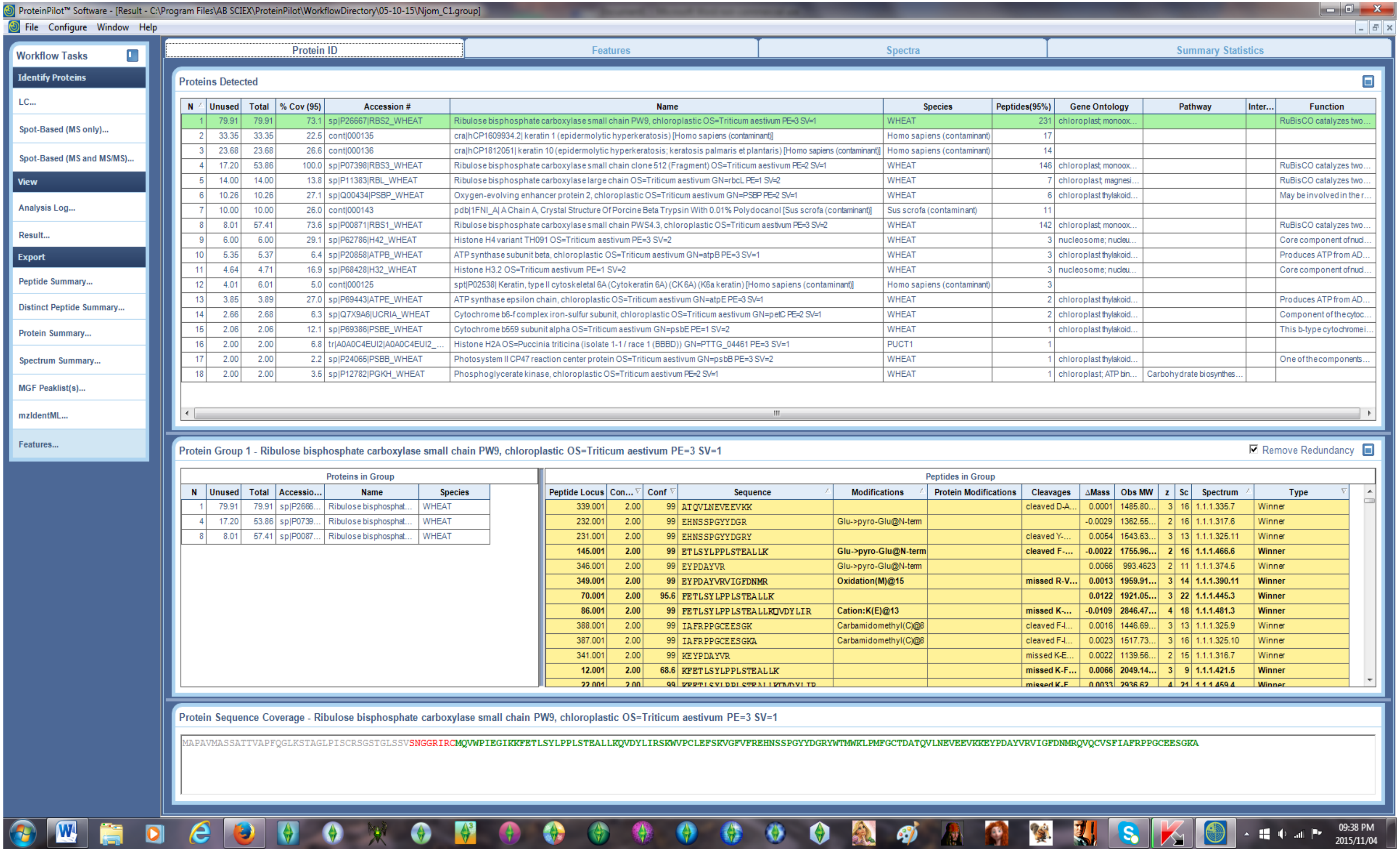


Proteins Detected

N	Unused	Total	% Cov (95)	Accession #	Name	Species	Peptides(95%)	Gene Ontology	Pathway	Inter...	Function
1	79.91	79.91	73.1	sp|P26667|RBS2_WHEAT	Ribulose bisphosphate carboxylase small chain PW9, chloroplastic OS=Triticum aestivum PE=3 SV=1	WHEAT	231	chloroplast; monoox...			RuBisCO catalyzes two...
2	33.35	33.35	22.5	cont|000135	cra|hCP1609934.2| keratin 1 (epidermolytic hyperkeratosis) [Homo sapiens (contaminant)]	Homo sapiens (contaminant)	17				
3	23.68	23.68	26.6	cont|000136	cra|hCP1812051| keratin 10 (epidermolytic hyperkeratosis; keratosis palmaris et plantaris) [Homo sapiens (contaminant)]	Homo sapiens (contaminant)	14				
4	17.20	53.86	100.0	sp|P07398|RBS3_WHEAT	Ribulose bisphosphate carboxylase small chain clone 512 (Fragment) OS=Triticum aestivum PE=2 SV=1	WHEAT	146	chloroplast; monoox...			RuBisCO catalyzes two...
5	14.00	14.00	13.8	sp|P11383|RBL_WHEAT	Ribulose bisphosphate carboxylase large chain OS=Triticum aestivum GN=rbcL PE=1 SV=2	WHEAT	7	chloroplast; magnesi...			RuBisCO catalyzes two...
6	10.26	10.26	27.1	sp|Q00434|PSBP_WHEAT	Oxygen-evolving enhancer protein 2, chloroplastic OS=Triticum aestivum GN=PSBP PE=2 SV=1	WHEAT	6	chloroplast thylakoid...			May be involved in the r...
7	10.00	10.00	26.0	cont|000143	pdb|1FNI_A| A Chain A, Crystal Structure Of Porcine Beta Trypsin With 0.01% Polydocanol [Sus scrofa (contaminant)]	Sus scrofa (contaminant)	11				
8	8.01	57.41	73.6	sp|P00871|RBS1_WHEAT	Ribulose bisphosphate carboxylase small chain PWS4.3, chloroplastic OS=Triticum aestivum PE=3 SV=2	WHEAT	142	chloroplast; monoox...			RuBisCO catalyzes two...
9	6.00	6.00	29.1	sp|P62786|H42_WHEAT	Histone H4 variant TH091 OS=Triticum aestivum PE=3 SV=2	WHEAT	3	nucleosome; nucleu...			Core component of nucl...
10	5.35	5.37	6.4	sp|P20858|ATPB_WHEAT	ATP synthase subunit beta, chloroplastic OS=Triticum aestivum GN=atpB PE=3 SV=1	WHEAT	3	chloroplast thylakoid...			Produces ATP from AD...
11	4.64	4.71	16.9	sp|P68428|H32_WHEAT	Histone H3.2 OS=Triticum aestivum PE=1 SV=2	WHEAT	3	nucleosome; nucleu...			Core component of nucl...
12	4.01	6.01	5.0	cont|000125	spt|P02538| Keratin, type II cytoskeletal 6A (Cytokeratin 6A) (CK 6A) (K6a keratin) [Homo sapiens (contaminant)]	Homo sapiens (contaminant)	3				
13	3.85	3.89	27.0	sp|P69443|ATPE_WHEAT	ATP synthase epsilon chain, chloroplastic OS=Triticum aestivum GN=atpE PE=3 SV=1	WHEAT	2	chloroplast thylakoid...			Produces ATP from AD...
14	2.66	2.68	6.3	sp|Q7X9A6|UCRIA_WHEAT	Cytochrome b6-f complex iron-sulfur subunit, chloroplastic OS=Triticum aestivum GN=petC PE=2 SV=1	WHEAT	2	chloroplast thylakoid...			Component of the cytoc...
15	2.06	2.06	12.1	sp|P69386|PSBE_WHEAT	Cytochrome b559 subunit alpha OS=Triticum aestivum GN=psbE PE=1 SV=2	WHEAT	1	chloroplast thylakoid...			This b-type cytochrome i...
16	2.00	2.00	6.8	tr|A0A0C4EUI2|A0A0C4EUI2_...	Histone H2A OS=Puccinia triticina (isolate 1-1 / race 1 (BBBD)) GN=PTTG_04461 PE=3 SV=1	PUCT1	1				
17	2.00	2.00	2.2	sp|P24065|PSBB_WHEAT	Photosystem II CP47 reaction center protein OS=Triticum aestivum GN=psbB PE=3 SV=2	WHEAT	1	chloroplast thylakoid...			One of the components...
18	2.00	2.00	3.5	sp|P12782|PGKH_WHEAT	Phosphoglycerate kinase, chloroplastic OS=Triticum aestivum PE=2 SV=1	WHEAT	1	chloroplast; ATP bin...	Carbohydrate biosynthes...		

Protein Group 1 - Ribulose bisphosphate carboxylase small chain PW9, chloroplastic OS=Triticum aestivum PE=3 SV=1

☑ Remove Redundancy

Proteins in Group

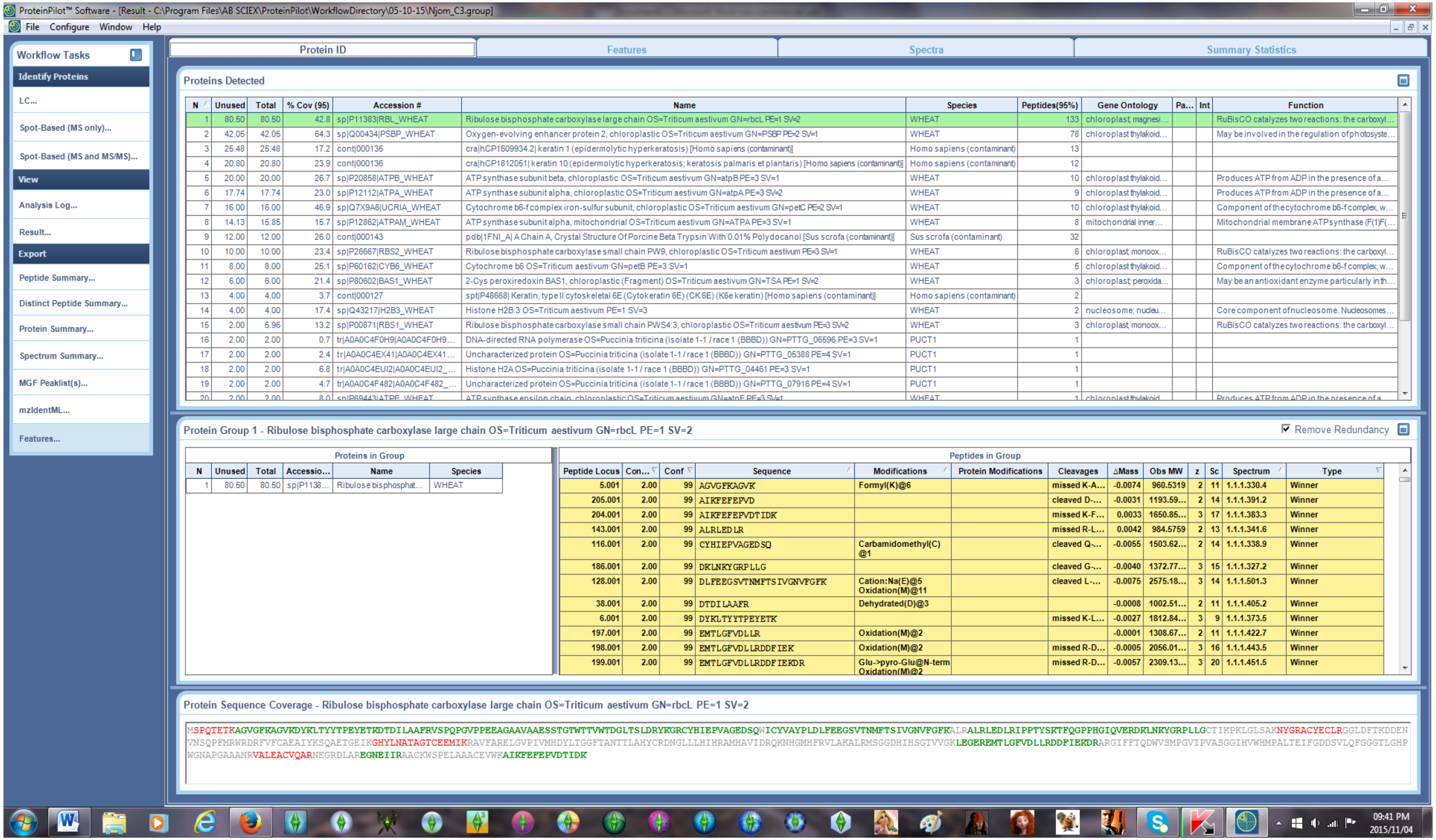
N	Unused	Total	Accessio...	Name	Species
1	79.91	79.91	sp|P2666...	Ribulose bisphosphat...	WHEAT
4	17.20	53.86	sp|P0739...	Ribulose bisphosphat...	WHEAT
8	8.01	57.41	sp|P0087...	Ribulose bisphosphat...	WHEAT

Peptides in Group

Peptide Locus	Con...	Conf	Sequence	Modifications	Protein Modifications	Cleavages	ΔMass	Obs MW	z	Sc	Spectrum	Type
339.001	2.00	99	ATQVLNEVEEVKK			cleaved D-A...	0.0001	1485.80...	3	16	1.1.1.335.7	Winner
232.001	2.00	99	EHNSSPGYYDGR	Glu->pyro-Glu@N-term			-0.0029	1362.55...	2	16	1.1.1.317.6	Winner
231.001	2.00	99	EHNSSPGYYDGRY			cleaved Y-...	0.0054	1543.63...	3	13	1.1.1.325.11	Winner
145.001	**2.00**	**99**	**ETLSYLPPLSTEALLK**	**Glu->pyro-Glu@N-term**		**cleaved F-...**	**-0.0022**	**1755.96...**	**2**	**16**	**1.1.1.466.6**	**Winner**
346.001	2.00	99	EYPDAYVR	Glu->pyro-Glu@N-term			0.0066	993.4623	2	11	1.1.1.374.5	Winner
349.001	**2.00**	**99**	**EYPDAYVRVIGFDNMR**	**Oxidation(M)@15**		**missed R-V...**	**0.0013**	**1959.91...**	**3**	**14**	**1.1.1.390.11**	**Winner**
70.001	**2.00**	**95.6**	**FETLSYLPPLSTEALLK**				**0.0122**	**1921.05...**	**3**	**22**	**1.1.1.445.3**	**Winner**
86.001	**2.00**	**99**	**FETLSYLPPLSTEALLKQVDYLIR**	**Cation:K(E)@13**		**missed K-...**	**-0.0109**	**2846.47...**	**4**	**18**	**1.1.1.481.3**	**Winner**
388.001	2.00	99	IAFRPPGCEESGK	Carbamidomethyl(C)@8		cleaved F-I...	0.0016	1446.69...	3	13	1.1.1.325.9	Winner
387.001	2.00	99	IAFRPPGCEESGKA	Carbamidomethyl(C)@8		cleaved F-I...	0.0023	1517.73...	3	16	1.1.1.325.10	Winner
341.001	2.00	99	KEYPDAYVR			missed K-E...	0.0022	1139.56...	2	15	1.1.1.316.7	Winner
12.001	**2.00**	**68.6**	**KFETLSYLPPLSTEALLK**			**missed K-F...**	**0.0066**	**2049.14...**	**3**	**9**	**1.1.1.421.5**	**Winner**
22.001	**2.00**	**99**	**KFETLSYLPPLSTEALLKQVDYLIR**			**missed K-F**	**0.0033**	**2936.62**	**4**	**21**	**1.1.1.459.4**	**Winner**

Protein Sequence Coverage - Ribulose bisphosphate carboxylase small chain PW9, chloroplastic OS=Triticum aestivum PE=3 SV=1

MAPAVMASSATTVAPFQGLKSTAGLPISCRSGSTGLSSVSNGGRIRCMQVWPIEGIKKFETLSYLPPLSTEALLKQVDYLIRSKWVPCLEFSKVGFVFREHNSSPGYYDGRYWTMWKLPMFGCTDATQVLNEVEEVKKEYPDAYVRVIGFDNMRQVQCVSFIAFRPPGCEESGKA



Proteins Detected

N	Unused	Total	% Cov (95)	Accession #	Name	Species	Peptides(95%)	Gene Ontology	Pa...	Int	Function
1	80.50	80.50	42.8	sp|P11383|RBL_WHEAT	Ribulose bisphosphate carboxylase large chain OS=Triticum aestivum GN=rbcL PE=1 SV=2	WHEAT	133	chloroplast; magnesi...			RuBisCO catalyzes two reactions: the carboxyl...
2	42.05	42.05	64.3	sp|Q00434|PSBP_WHEAT	Oxygen-evolving enhancer protein 2, chloroplastic OS=Triticum aestivum GN=PSBP PE=2 SV=1	WHEAT	78	chloroplast thylakoid...			May be involved in the regulation of photosyste...
3	25.48	25.48	17.2	cont|000135	cra|hCP1609934.2| keratin 1 (epidermolytic hyperkeratosis) [Homo sapiens (contaminant)]	Homo sapiens (contaminant)	13				
4	20.80	20.80	23.9	cont|000136	cra|hCP1812051| keratin 10 (epidermolytic hyperkeratosis; keratosis palmaris et plantaris) [Homo sapiens (contaminant)]	Homo sapiens (contaminant)	12				
5	20.00	20.00	26.7	sp|P20858|ATPB_WHEAT	ATP synthase subunit beta, chloroplastic OS=Triticum aestivum GN=atpB PE=3 SV=1	WHEAT	10	chloroplast thylakoid...			Produces ATP from ADP in the presence of a...
6	17.74	17.74	23.0	sp|P12112|ATPA_WHEAT	ATP synthase subunit alpha, chloroplastic OS=Triticum aestivum GN=atpA PE=3 SV=2	WHEAT	9	chloroplast thylakoid...			Produces ATP from ADP in the presence of a...
7	16.00	16.00	46.9	sp|Q7X9A6|UCRIA_WHEAT	Cytochrome b6-f complex iron-sulfur subunit, chloroplastic OS=Triticum aestivum GN=petC PE=2 SV=1	WHEAT	10	chloroplast thylakoid...			Component of the cytochrome b6-f complex, w...
8	14.13	15.85	15.7	sp|P12862|ATPAM_WHEAT	ATP synthase subunit alpha, mitochondrial OS=Triticum aestivum GN=ATPA PE=3 SV=1	WHEAT	8	mitochondrial inner...			Mitochondrial membrane ATP synthase (F(1)F(...
9	12.00	12.00	26.0	cont|000143	pdb|1FNI_A| A Chain A, Crystal Structure Of Porcine Beta Trypsin With 0.01% Polydocanol [Sus scrofa (contaminant)]	Sus scrofa (contaminant)	32				
10	10.00	10.00	23.4	sp|P26667|RBS2_WHEAT	Ribulose bisphosphate carboxylase small chain PW9, chloroplastic OS=Triticum aestivum PE=3 SV=1	WHEAT	6	chloroplast; monoox...			RuBisCO catalyzes two reactions: the carboxyl...
11	8.00	8.00	25.1	sp|P60162|CYB6_WHEAT	Cytochrome b6 OS=Triticum aestivum GN=petB PE=3 SV=1	WHEAT	5	chloroplast thylakoid...			Component of the cytochrome b6-f complex, w...
12	6.00	6.00	21.4	sp|P80602|BAS1_WHEAT	2-Cys peroxiredoxin BAS1, chloroplastic (Fragment) OS=Triticum aestivum GN=TSA PE=1 SV=2	WHEAT	3	chloroplast; peroxida...			May be an antioxidant enzyme particularly in th...
13	4.00	4.00	3.7	cont|000127	spt|P48668| Keratin, type II cytoskeletal 6E (Cytokeratin 6E) (CK 6E) (K6e keratin) [Homo sapiens (contaminant)]	Homo sapiens (contaminant)	2				
14	4.00	4.00	17.4	sp|Q43217|H2B3_WHEAT	Histone H2B.3 OS=Triticum aestivum PE=1 SV=3	WHEAT	2	nucleosome; nucleu...			Core component of nucleosome. Nucleosomes...
15	2.00	5.96	13.2	sp|P00871|RBS1_WHEAT	Ribulose bisphosphate carboxylase small chain PWS4.3, chloroplastic OS=Triticum aestivum PE=3 SV=2	WHEAT	3	chloroplast; monoox...			RuBisCO catalyzes two reactions: the carboxyl...
16	2.00	2.00	0.7	tr|A0A0C4F0H9|A0A0C4F0H9...	DNA-directed RNA polymerase OS=Puccinia triticina (isolate 1-1 / race 1 (BBBD)) GN=PTTG_06596 PE=3 SV=1	PUCT1	1				
17	2.00	2.00	2.4	tr|A0A0C4EX41|A0A0C4EX41...	Uncharacterized protein OS=Puccinia triticina (isolate 1-1 / race 1 (BBBD)) GN=PTTG_05388 PE=4 SV=1	PUCT1	1				
18	2.00	2.00	6.8	tr|A0A0C4EUI2|A0A0C4EUI2_...	Histone H2A OS=Puccinia triticina (isolate 1-1 / race 1 (BBBD)) GN=PTTG_04461 PE=3 SV=1	PUCT1	1				
19	2.00	2.00	4.7	tr|A0A0C4F482|A0A0C4F482_...	Uncharacterized protein OS=Puccinia triticina (isolate 1-1 / race 1 (BBBD)) GN=PTTG_07916 PE=4 SV=1	PUCT1	1				
20	2.00	2.00	8.0	sp|P69443|ATPE_WHEAT	ATP synthase epsilon chain, chloroplastic OS=Triticum aestivum GN=atpE PE=3 SV=1	WHEAT	1	chloroplast thylakoid			Produces ATP from ADP in the presence of a

Protein Group 1 - Ribulose bisphosphate carboxylase large chain OS=Triticum aestivum GN=rbcL PE=1 SV=2

☑ Remove Redundancy

Proteins in Group

N	Unused	Total	Accessio...	Name	Species
1	80.50	80.50	sp|P1138...	Ribulose bisphosphat...	WHEAT

Peptides in Group

Peptide Locus	Con...	Conf	Sequence	Modifications	Protein Modifications	Cleavages	ΔMass	Obs MW	z	Sc	Spectrum	Type
5.001	2.00	99	AGVGFKAGVK	Formyl(K)@6		missed K-A...	-0.0074	960.5319	2	11	1.1.1.330.4	Winner
205.001	2.00	99	AIKFEFEPVD			cleaved D-...	-0.0031	1193.59...	2	14	1.1.1.391.2	Winner
204.001	2.00	99	AIKFEFEPVDTIDK			missed K-F...	0.0033	1650.85...	3	17	1.1.1.383.3	Winner
143.001	2.00	99	ALRLEDLR			missed R-L...	0.0042	984.5759	2	13	1.1.1.341.6	Winner
116.001	2.00	99	CYHIEPVAGEDSQ	Carbamidomethyl(C)@1		cleaved Q-...	-0.0055	1503.62...	2	14	1.1.1.338.9	Winner
186.001	2.00	99	DKLNKYGRPLLG			cleaved G-...	-0.0040	1372.77...	3	15	1.1.1.327.2	Winner
128.001	2.00	99	DLFEEGSVTNMFTSIVGNVFGFK	Cation:Na(E)@5 Oxidation(M)@11		cleaved L-...	-0.0075	2575.18...	3	14	1.1.1.501.3	Winner
38.001	2.00	99	DTDILAAFR	Dehydrated(D)@3			-0.0008	1002.51...	2	11	1.1.1.405.2	Winner
6.001	2.00	99	DYKLTYYTPEYETK			missed K-L...	-0.0027	1812.84...	3	9	1.1.1.373.5	Winner
197.001	2.00	99	EMTLGFVDLLR	Oxidation(M)@2			-0.0001	1308.67...	2	11	1.1.1.422.7	Winner
198.001	2.00	99	EMTLGFVDLLRDDFIEK	Oxidation(M)@2		missed R-D...	-0.0005	2056.01...	3	16	1.1.1.443.5	Winner
199.001	2.00	99	EMTLGFVDLLRDDFIEKDR	Glu->pyro-Glu@N-term Oxidation(M)@2		missed R-D...	-0.0057	2309.13...	3	20	1.1.1.451.5	Winner

Protein Sequence Coverage - Ribulose bisphosphate carboxylase large chain OS=Triticum aestivum GN=rbcL PE=1 SV=2

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MSPQTETKAGVGFKAGVKDYKLTYYTPEYETKDTDILAAFRVSPQPGVPPEEAGAAVAAESSTGTWTTVWTDGLTSLDRYKGRCYHIEPVAGEDSQWICYVAYPLDLFEEGSVTNMFTSIVGNVFGFKALRALRLEDLRIPPTYSKTFQGPPHGIQVERDKLNKYGRPLLGCTIKPKLGLSAKNYGRACYECLRGGLDFTKDDEN
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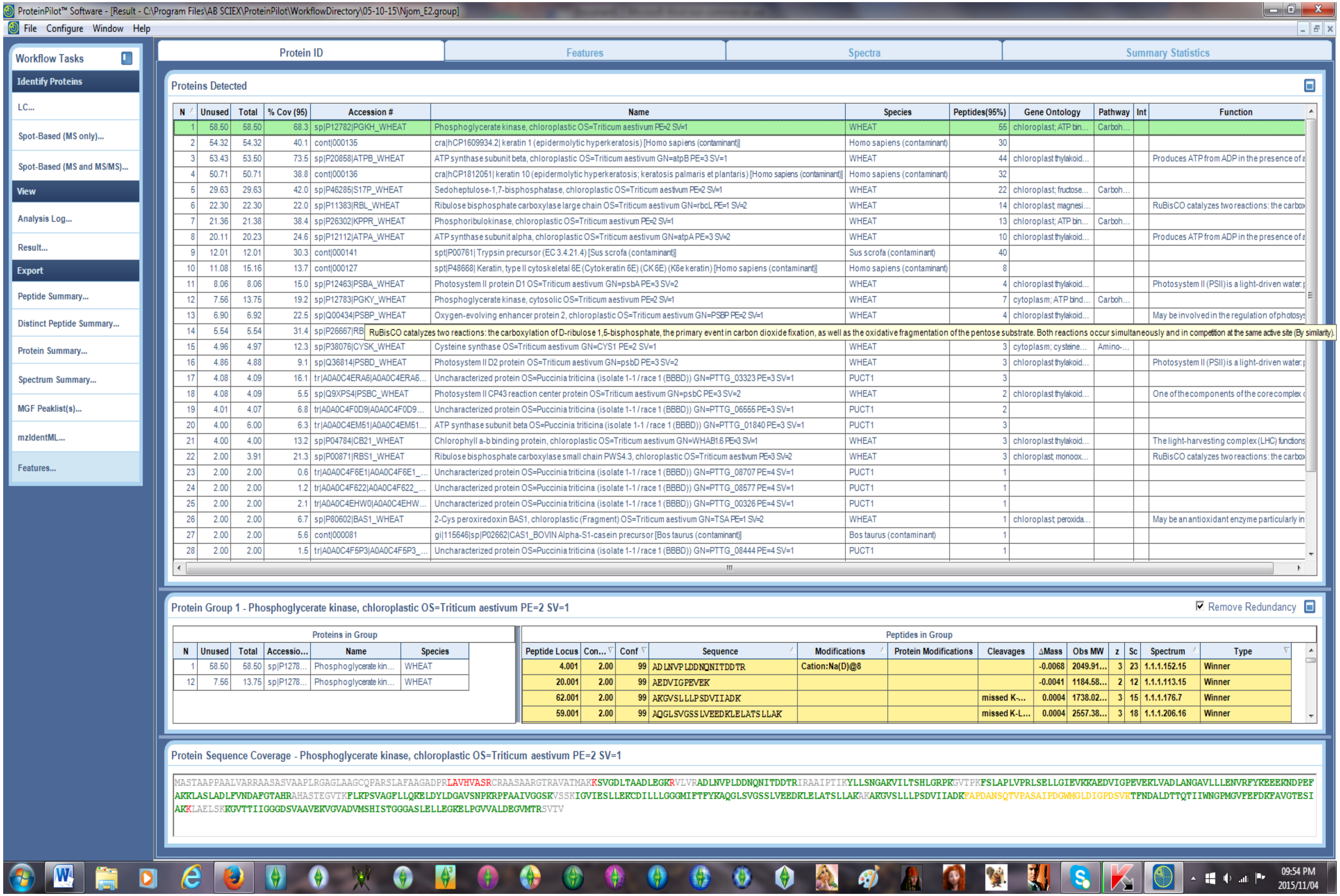

Proteins Detected

N	Unused	Total	% Cov (95)	Accession #	Name	Species	Peptides(95%)	Gene Ontology	Pathway	Int	Function
1	58.50	58.50	68.3	sp|P12782|PGKH_WHEAT	Phosphoglycerate kinase, chloroplastic OS=Triticum aestivum PE=2 SV=1	WHEAT	55	chloroplast; ATP bin...	Carboh...		
2	54.32	54.32	40.1	cont|000135	cra|hCP1609934.2| keratin 1 (epidermolytic hyperkeratosis) [Homo sapiens (contaminant)]	Homo sapiens (contaminant)	30				
3	53.43	53.50	73.5	sp|P20858|ATPB_WHEAT	ATP synthase subunit beta, chloroplastic OS=Triticum aestivum GN=atpB PE=3 SV=1	WHEAT	44	chloroplast thylakoid...			Produces ATP from ADP in the presence of a
4	50.71	50.71	38.8	cont|000136	cra|hCP1812051| keratin 10 (epidermolytic hyperkeratosis; keratosis palmaris et plantaris) [Homo sapiens (contaminant)]	Homo sapiens (contaminant)	32				
5	29.63	29.63	42.0	sp|P46285|S17P_WHEAT	Sedoheptulose-1,7-bisphosphatase, chloroplastic OS=Triticum aestivum PE=2 SV=1	WHEAT	22	chloroplast; fructose...	Carboh...		
6	22.30	22.30	22.0	sp|P11383|RBL_WHEAT	Ribulose bisphosphate carboxylase large chain OS=Triticum aestivum GN=rbcL PE=1 SV=2	WHEAT	14	chloroplast; magnesi...			RuBisCO catalyzes two reactions: the carbox
7	21.36	21.38	38.4	sp|P26302|KPPR_WHEAT	Phosphoribulokinase, chloroplastic OS=Triticum aestivum PE=2 SV=1	WHEAT	13	chloroplast; ATP bin...	Carboh...		
8	20.11	20.23	24.6	sp|P12112|ATPA_WHEAT	ATP synthase subunit alpha, chloroplastic OS=Triticum aestivum GN=atpA PE=3 SV=2	WHEAT	10	chloroplast thylakoid...			Produces ATP from ADP in the presence of a
9	12.01	12.01	30.3	cont|000141	spt|P00761| Trypsin precursor (EC 3.4.21.4) [Sus scrofa (contaminant)]	Sus scrofa (contaminant)	40				
10	11.08	15.16	13.7	cont|000127	spt|P48668| Keratin, type II cytoskeletal 6E (Cytokeratin 6E) (CK 6E) (K6e keratin) [Homo sapiens (contaminant)]	Homo sapiens (contaminant)	8				
11	8.06	8.06	15.0	sp|P12463|PSBA_WHEAT	Photosystem II protein D1 OS=Triticum aestivum GN=psbA PE=3 SV=2	WHEAT	4	chloroplast thylakoid...			Photosystem II (PSII) is a light-driven water p
12	7.56	13.75	19.2	sp|P12783|PGKY_WHEAT	Phosphoglycerate kinase, cytosolic OS=Triticum aestivum PE=2 SV=1	WHEAT	7	cytoplasm; ATP bind...	Carboh...		
13	6.90	6.92	22.5	sp|Q00434|PSBP_WHEAT	Oxygen-evolving enhancer protein 2, chloroplastic OS=Triticum aestivum GN=PSBP PE=2 SV=1	WHEAT	4	chloroplast thylakoid...			May be involved in the regulation of photosys
14	5.54	5.54	31.4	sp|P26667|RB							
15	4.96	4.97	12.3	sp|P38076|CYSK_WHEAT	Cysteine synthase OS=Triticum aestivum GN=CYS1 PE=2 SV=1	WHEAT	3	cytoplasm; cysteine...	Amino-...		
16	4.86	4.88	9.1	sp|Q36814|PSBD_WHEAT	Photosystem II D2 protein OS=Triticum aestivum GN=psbD PE=3 SV=2	WHEAT	3	chloroplast thylakoid...			Photosystem II (PSII) is a light-driven water p
17	4.08	4.09	16.1	tr|A0A0C4ERA6|A0A0C4ERA6...	Uncharacterized protein OS=Puccinia triticina (isolate 1-1 / race 1 (BBBD)) GN=PTTG_03323 PE=3 SV=1	PUCT1	3				
18	4.08	4.09	5.5	sp|Q9XPS4|PSBC_WHEAT	Photosystem II CP43 reaction center protein OS=Triticum aestivum GN=psbC PE=3 SV=2	WHEAT	2	chloroplast thylakoid...			One of the components of the core complex o
19	4.01	4.07	6.8	tr|A0A0C4F0D9|A0A0C4F0D9...	Uncharacterized protein OS=Puccinia triticina (isolate 1-1 / race 1 (BBBD)) GN=PTTG_06555 PE=3 SV=1	PUCT1	2				
20	4.00	6.00	6.3	tr|A0A0C4EM51|A0A0C4EM51...	ATP synthase subunit beta OS=Puccinia triticina (isolate 1-1 / race 1 (BBBD)) GN=PTTG_01840 PE=3 SV=1	PUCT1	3				
21	4.00	4.00	13.2	sp|P04784|CB21_WHEAT	Chlorophyll a-b binding protein, chloroplastic OS=Triticum aestivum GN=WHAB1.6 PE=3 SV=1	WHEAT	3	chloroplast thylakoid...			The light-harvesting complex (LHC) functions
22	2.00	3.91	21.3	sp|P00871|RBS1_WHEAT	Ribulose bisphosphate carboxylase small chain PWS4.3, chloroplastic OS=Triticum aestivum PE=3 SV=2	WHEAT	3	chloroplast; monoox...			RuBisCO catalyzes two reactions: the carbox
23	2.00	2.00	0.6	tr|A0A0C4F6E1|A0A0C4F6E1_...	Uncharacterized protein OS=Puccinia triticina (isolate 1-1 / race 1 (BBBD)) GN=PTTG_08707 PE=4 SV=1	PUCT1	1				
24	2.00	2.00	1.2	tr|A0A0C4F622|A0A0C4F622_...	Uncharacterized protein OS=Puccinia triticina (isolate 1-1 / race 1 (BBBD)) GN=PTTG_08577 PE=4 SV=1	PUCT1	1				
25	2.00	2.00	2.1	tr|A0A0C4EHW0|A0A0C4EHW...	Uncharacterized protein OS=Puccinia triticina (isolate 1-1 / race 1 (BBBD)) GN=PTTG_00326 PE=4 SV=1	PUCT1	1				
26	2.00	2.00	6.7	sp|P80602|BAS1_WHEAT	2-Cys peroxiredoxin BAS1, chloroplastic (Fragment) OS=Triticum aestivum GN=TSA PE=1 SV=2	WHEAT	1	chloroplast; peroxida...			May be an antioxidant enzyme particularly in
27	2.00	2.00	5.6	cont|000081	gi|115646|sp|P02662|CAS1_BOVIN Alpha-S1-casein precursor [Bos taurus (contaminant)]	Bos taurus (contaminant)	1				
28	2.00	2.00	1.5	tr|A0A0C4F5P3|A0A0C4F5P3_...	Uncharacterized protein OS=Puccinia triticina (isolate 1-1 / race 1 (BBBD)) GN=PTTG_08444 PE=4 SV=1	PUCT1	1				

RuBisCO catalyzes two reactions: the carboxylation of D-ribulose 1,5-bisphosphate, the primary event in carbon dioxide fixation, as well as the oxidative fragmentation of the pentose substrate. Both reactions occur simultaneously and in competition at the same active site (By similarity).

Protein Group 1 - Phosphoglycerate kinase, chloroplastic OS=Triticum aestivum PE=2 SV=1

☑ Remove Redundancy

Proteins in Group

N	Unused	Total	Accessio...	Name	Species
1	58.50	58.50	sp|P1278...	Phosphoglycerate kin...	WHEAT
12	7.56	13.75	sp|P1278...	Phosphoglycerate kin...	WHEAT

Peptides in Group

Peptide Locus	Con...	Conf	Sequence	Modifications	Protein Modifications	Cleavages	ΔMass	Obs MW	z	Sc	Spectrum	Type
4.001	2.00	99	ADLNVPLDDNQNITDDTR	Cation:Na(D)@8			-0.0068	2049.91...	3	23	1.1.1.152.15	Winner
20.001	2.00	99	AEDVIGPEVEK				-0.0041	1184.58...	2	12	1.1.1.113.15	Winner
62.001	2.00	99	AKGVSLLLPSDVIIADK			missed K-...	0.0004	1738.02...	3	15	1.1.1.176.7	Winner
59.001	2.00	99	AQGLSVGSSLVEEDKLELATSLLAK			missed K-L...	0.0004	2557.38...	3	18	1.1.1.206.16	Winner

Protein Sequence Coverage - Phosphoglycerate kinase, chloroplastic OS=Triticum aestivum PE=2 SV=1

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MASTAAPPAALVARRAASASVAAPLRGAGLAAGCQPARSLAFAAGADPRLAVHVASRCRAASAARGTRAVATMAKKSVGDLTAADLEGKRVLVRADLNVPLDDNQNITDDTRIRAAIPTIKYLLSNGAKVILTSHLGRPKGVTPKFSLAPLVPRLSELLGIEVKKAEDVIGPEVEKLVADLANGAVLLLENVRFYKEEEKNDPEF
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