Potential role of photosynthetic and metabolic related proteins in the resistance mechanism of Tugela Dn against Russian Wheat Aphid-SA2.

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ABSTRACT

Continued increase in the world population increases the demand for food, which requires active intervention to ensure food security. Furthermore, greenhouse gases have resulted in global warming which has triggered climate change, with the consequence that food production systems have been negatively affected and this imparts further risk to food security. The agricultural sector, and thus global food security, therefore is directly affected by these changing climatic conditions since they result in an increase of insect outbreaks as well as related resistant breaking pathogens. The effect of carbon dioxide (CO_2) on cereal's most economically significant insect pests, the aphid, is not well researched. Due to the destructive nature of D. noxia's feeding behaviour on crops, several methods have been devised to control the damages. In South Africa, the first wheat resistant cultivar, Tugela Dn which contained the *Dn* resistance gene was released in 1992. Initially it was reported that resistant cultivars reduce aphid population by inhibiting Russian Wheat Aphid (RWA) growth and reproduction. Recently, the presence of resistance breaking RWA biotypes in South Africa was confirmed and is thought to be more virulent on existing RWA-resistant wheat lines. This study focussed on gaining a greater understanding of the resistance mechanisms activated by the Russian Wheat Aphid resistance gene *Dn* (*Dn*1) and whether RWA-SA2 overcomes the resistance response of Tugela Dn with the aim of improving agronomical traits of wheat plants. Initial reports showed that RWA-SA2 not only bred faster but also caused more damage to wheat lines than did RWA-SA1. RWA-SA2 appeared unaffected by the *Dn*1 resistance gene and posed a serious threat to small grain production in South Africa. In the first part of this study, we evaluated whether RWA-SA2 damages/compromises the photosynthetic mechanisms of wheat by measuring chlorophyll fluorescence of infested RWA-SA2 susceptible and resistant wheat cultivars as well as whether there are any changes in stomatal conductance. Results obtained indicate that although both wheat isolines (Tugela and Tugela Dn) exhibited aphid injury, Tugela as expected appeared to be affected more and at an earlier stage. It would appear that the chlorophyll concentration in the uninfested Tugela leaves were significantly higher than RWA-SA2 infested Tugela leaves from 120 hours onwards. Tugela Dn infested leaves on the other hand appeared to have thrived quite well with negligible chlorophyll concentration loss.

Stomatal conductance was enhanced considerably in Tugela by RWA-SA2 feeding suggesting increased stomatal apertures. However, stomatal conductance dropped after day 7 in Tugela which is a result from damage to the leaf tissues. The second part of this study focussed on identifying exclusive expressed proteins during RWA-SA2 infestation on RWA-SA1 susceptible and resistant wheat cultivars as well as to identify possible signalling pathways induced in cereals. The Tugela wheat cultivar provided more evidentiary support that during the initial hours of RWA-SA2 phloem feeding, several proteins were down-regulated that could possibly indicate an initial response to phloem feeding by "switching off" the plants metabolic mechanisms and preventing the flow of nutrients to the sieve elements for aphids and/or other phloem feeders to benefit. Exclusively identified proteins were largely involved in photosynthesis, metabolism and stress suggesting that the rate of incorporation and/or exportation of photosynthetic products decline, becoming restricted by feedback inhibition. It would appear that the pathways identified function in parallel to capitalize on more defense efforts rather than resistance against RWA-SA2 as these pathways seem to be interlinked. This study therefore provides evidence that Tugela Dn, seem to counteract deleterious effects of aphid (RWA-SA2) herbivory through up-regulation and faster regeneration of photosynthetic related molecule and does respond in a highly specific manner to infestation with RWA-SA2 by inducing unique pathways. It would also appear that RWA-SA2 partially overcomes the resistance response of Tugela Dn against RWA-SA1.

DECLARATION

I ADRIAN MARK ABRAHAMS declare that this dissertation titled "**Potential role of photosynthetic and metabolic related proteins in the resistance mechanism of Tugela Dn against Russian Wheat Aphid-SA2"** submitted for the award of the Doctor of Philosophy degree in Biochemistry at the University of Fort Hare is my own work that has never been submitted for any other degree at this university or any other university.

____

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I ADRIAN MARK ABRAHAMS, student number 200702453 hereby declare that I am fully aware of the University of Fort Hare policy on plagiarism and I have taken every precaution to comply with the regulations

Signature: ___________________

I ADRIAN MARK ABRAHAMS, student number 200702453 hereby declare that I am fully aware of the University of Fort Hare policy on research ethics and have taken every precaution to comply with the regulations. There was no need for ethical clearance.

Signature: ___________________

I declare that this is my own work

DEDICATION

This thesis is dedicated to Mother (Jackie); siblings (Andre and Belisha), the loving memory of my father, the late Andrew (Billy) Abrahams as well as to my grandmother, the late Lydia (mama Toeksie) Strydom. I suppose growing up wasn't all that easy but thank you for nurturing me and giving me the love I needed throughout the years. You ALL BELIEVE in me and knew I would get this far in life and continue to strive for even better in life.

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LIST OF ABBREVIATIONS

ABA	**Abscisic acid**
ACN	**Acetonitrile**
NH_4HCO_3	**Ammonium bicarbonate**
ARC	**Agricultural Research Council**
IAA	**Iodoacetamide**
BSA	**Bovine Serum Albumin**
CO_2	**Carbon dioxide**
D.noxia	***Diuraphis noxia***
DTT	**Dithiothreitol**
ESI	**Electron Spray Ionizer**
F_0	**non-variable fluorescence**
FAO	**Food and Agriculture Organization**
F_m	**maximum fluorescence**
FA	**Formic acid**
F_v	**variable fluorescence**
F_v/F_m	**photochemical efficiency of photosystem II**
GC	**gas chromatography**
GA	**Gibberellic acid**
H_2O	**water**
HPLC	**High Performance Liquid Chromatography**
H_2O_2	**Hydrogen peroxide**
HR	**hypersensitive response**
IPG Strips	**immobilized pH gradient strips**
IAA	**Iodoacetamide**
IPCC	**Intergovernmental panel on climate change**
IEF	**isoelectric focusing**
JA	**Jasmonic Acid**
LCMS	**Liquid Chromatography Mass Spectrometry**
MS	**mass spectrometry**
MeOH	**Methanol**
NO	**Nitric oxide**

PAMP's	**Pathogen-associated molecular patterns**
PRR	**Pattern recognition receptors**
PDA	**photodiode array**
DWP	**Protein Low-Bind Deep well plates**
Q_A	**Quinone A**
Q_B	**Quinone B**
ROS	**Reactive oxygen species**
RWA-SA2	**Russian Wheat Aphid South African biotype 2**
SA	**Salicylic acid**
SDS-PAGE	**sodium dodecyl sulphate polyacrylamide gel electrophoresis**
SSP	**Standard Spot**
SAR	**systemic acquired resistance**
2DE	**Two dimensional gel electrophoresis**
pI	**Isoelectric point**
kDa	**Kilo Daltons**
NADPH	**Nicotinamide adenine dinucleotide phosphate**
ATP	**Adenosine triphosphate**
NADH	**Nicotinamide adenine dinucleotide**
DHN	**Dehydrins**
LEA	**Late Embryogenesis Abundant**
***DN*A**	**Deoxyribonucleic acid**
LHC II	**Light Harvesting Complex II**
PSI	**photosystem I**
PSII	**photosystem II**
ROS	**Reactive Oxygen Species**

CHAPTER 1

Literature Review

1.1) Introduction

Research over the past several years has shown that global warming is unequivocal with evidence being presented by observations seen in the increasing global air temperature and the melting of snow and ice, which inevitably leads to rising sea levels as well as increases in ocean temperatures. Furthermore over the past two decades there has been a steady drop in atmospheric carbon dioxide (CO_2) and greenhouse gases (IPCC, 2007). Increases in greenhouse gases have also been linked to widespread global changes in rainfall patterns. This rate of global warming and climate change is expected to continue increasing if no mitigation factors or interventions are put in place by industries, farmers and private households in order to alleviate the emission of greenhouse gases (Raupach *et al.*, 2007). Temperature controls the rate of a plant's metabolism which ultimately influences the final yield of agricultural crops (Hay and Walker, 1989).

The agricultural sector, and thus global food security, therefore is directly affected by these changing climatic conditions since they result in an increase of insect outbreaks as well as related resistant breaking pathogens. The effect of CO_2 on the most economically significant insect pests of cereals, the aphid, is not well researched. However, it is well known that increased CO_2 levels increase the growth rate of C_3 plants and affect insect metabolism, therefore it is expected that the interaction between aphids and their host plants will alter under various CO_2 conditions. Preliminary studies by Jimoh *et al.* (2012) and Mudondo (2014) have indicated that varying CO_2 levels may result in certain aphids breaking the resistance of selected wheat cultivars as well as causing increased damage to barley crops, thereby seriously affecting crop yields and food security.

This study focusses on gaining a greater understanding of the resistance mechanisms activated by the Russian Wheat Aphid resistance gene *Dn* (*Dn*1) and whether RWA-SA2 overcomes the resistance response of Tugela Dn with the aim of improving agronomical traits of wheat plants. This dissertation will then explore the effect of ambient CO_2 on the behaviour and fecundity of the aphid species and how the wheat proteome is altered. In chapter two this dissertation will investigate changes on chlorophyll fluorescence induction kinetics as well as the significance of photosynthesis and photosynthesis related gene products (Chapter Four).

Research shows that the world population is currently obtaining more than 50% of its vital calories from crops such as wheat, maize, Oryza sativa and potato (Webb, 2000), with wheat being the most widely produced cereal crop in the world (Curtis, 2002). This is mainly due to the great versatility of wheat which may be used in the production of dough, making of alcoholic beverages, as well as being fed to animals as fodder. Plants encounter various biotic and abiotic challenges, which usually lead to interactions between the plant and the eliciting agent. Typical abiotic stress factors encountered by wheat producers can include aluminium toxicity due to acid soils and pre-harvest sprouting after wet spells during wheat ripening. Biotic stresses include plant diseases and a number of insect pests. Of these insect pests, the Russian Wheat Aphid has been found to be the most economically important pest of wheat in South Africa (Walters, 1984). This review aims to present an overview on the host plant, wheat, a detailed and thorough perspective on how plants generally respond to various stress factors as well as the interaction of the Russian Wheat Aphid with its host plant.

1.2) Wheat

Wheat is an essential crop and arguably the most versatile of all commercial crops. Some of these versatile products include: making dough, feed for animals, a stabiliser/bulking agent in processed food as well as the generation of biofuels to mention a few (Hogy and Fangmeier, 2008). It originated from the Near East or Western Asia and is reported to have first been grown in the United States in 1602 on an island off the Massachusetts coast (Gibson and Benson, 2002). Wheat makes a substantial contribution to the economy of many countries and its worldwide trade is higher than any other crop. Reports show that the crop provides approximately 35% of the staple food and about 20% of the calories for the worlds' population (FAO, 1998; Gibson and Benson, 2002). Wheat's role as a staple food has resulted in many studies on its responses to both abiotic and biotic stresses as well as the focus of breeding programmes.

1.3) Plant responses to various stress factors

Plant productivity and growth is adversely affected by various stress factors. They are frequently exposed to a variety of stress signals such as cold, heat, salinity, drought, pathogens, and herbivores. Stress in its physical term may be defined as the mechanical force applied to an object. As plants are sessile, it is quite hard to measure the exact force exerted by various stress conditions and therefore in biological terms it is difficult to define a stress. A biological condition which acts as a stress for one plant may serve as an optimum for another plant. Therefore, the most practical definition one can give to a biological stress would be an adverse condition which inhibits the normal functioning and well-being of a biological system such as plants. Various stress factors such as abiotic stress factors are the centre of much research because of their potential impact on the yield and quality of agriculture products (Ligang *et al.*, 2012). Both abiotic and biotic stresses cause major crop damage and result in significant crop losses annually worldwide (Rushton and Somssich, 1998). The response of plants to these stresses can either take an active or passive form. An active defense response involves a suitable and rapid response when a stress is detected while a passive response is more delayed and takes more time to be activated (Hammond-Kosack and Jones, 2000). When plants perceive a stress, a response is initiated that activates specific signal transduction pathways that facilitates the generation of appropriate physical and physiological responses. Various signalling pathways are involved in different stress responses with multiple cross-talks occurring, resulting in a highly complex myriad of networks (Maleck and Dietrich, 1999).

1.3.1) Abiotic Stress Responses

1.3.1.1) Introduction

Abiotic stress factors are any type of environmental condition that adversely impacts plant productivity, development and survival and include extreme low or high temperatures, drought, and variations in soil pH, nutrient supply, extreme light intensity and mechanical damage (Boscaiu *et al.*, 2008). Plants have a unique set of temperature requirements which are optimal for their proper growth and development. A certain set of optimum conditions for one plant may be stressful for another plant.

Essential crops such as wheat and Oryza sativa have been found to be sensitive to all the abiotic stress conditions mentioned earlier, all of which have a negative impact on yield (Atienza *et al.*, 2004).

1.3.1.2) Drought

Drought is a serious problem for agriculture worldwide and may arise as a result of two conditions, either due to excess or shortage of water. An example of surplus water is flooding which results in reduced oxygen supply to plant roots. This negatively impacts the uptake of nutrients from the soil as well as respiration. Water shortage is usually referred to as drought stress, leading to reduced vegetative growth and survival. Removal of water from plants also causes the phospholipid bilayer to shrink and become exceptionally porous. Stress within this bilayer may also result in displacement of membrane proteins, loss of enzyme activity and disruption of cellular compartmentalization (Liu and Zhu, 1998) leading to tougher foliage. There have been reports that prolonged or severe drought may also trigger insect outbreaks in forests (Jactel *et al.*, 2012). There have also been reports that drought stress causes changes in the expression levels of LEA/Dehydrin genes and molecular chaperones (Hanin *et al*., 2011). This type of stress also activates enzymes which are involved in the synthesis and removal of reactive oxygen species (ROS) (Zhu, 2002; Cushman and Bohnert, 2000). Phloem feeder's, such as aphids, response to drought is not well understood but work done by Vickers (2011) has shown that under drought conditions, sieve elements increase in amino acid concentration and proportion of small nitrogenous molecules which will ultimately be of benefit to aphids since they are limited by available nitrogen in their diet. Aslam *et al*. (2012) investigated the effect of summer drought on the bird cherry-oat aphid, *Rhopalosiphum padi L*, feeding on barley. Both the drought and the aphids reduced the barley dry mass by 33% and 39% respectively as well as leaf area and nitrogen concentration by 13% and 28% respectively. The aphid population appeared to be unaffected by drought although the population demography changed significantly.

1.3.1.3) Temperature

Temperature controls the rate of plant metabolic processes that eventually influence agricultural production (Hay and Walker, 1989). It has been predicted that there will be an increase in 1.4 – 5.8°C in the global mean temperature in the next century and this will impact the biology of each species in various ways (Houghton *et al.*, 2001). The productivity of important agricultural crops is drastically reduced when they experience short episodes of high temperatures during the reproductive period. Agricultural production, and thus global food security, is directly affected by global warming (Fischer *et al.*, 2005; Schmidhuber and Tubiello, 2007; Ainsworth and Ort, 2010). It has also been reported that high temperatures play a detrimental role in the population and development of some aphid species such as the rose grain aphid *Metopolophium dirhodum* (Zhou and Carter, 1992). High temperatures can also increase the risk of drought, limit the rate of photosynthesis and reduce light interception by accelerating phenological development (Tubiello *et al.*, 2007). Continuous higher temperatures may result in heat stress, subsequently affecting protein folding, and other membrane complexes. Chaperone functions may be altered especially heat-shock proteins (HSP's) as they also play a role in protein folding (Sanmiya *et al.*, 2004).

Cold as a stress factor is associated with noteworthy alterations in energy metabolism that cause a decrease in the rate of enzyme catalysed reactions resulting in metabolic imbalances associated with a reduced water uptake leading to cellular dehydration (Thomashow, 1999; Ruelland *et al.*, 2009). Cold stress aspects profoundly affect plant responses. The impact of cold in proteome abundance has been studied in *Arabidopsis thaliana* (Bae *et al.*, 2003; Amme *et al.*, 2006), *Thellungiella halophila* (Gao *et al.*, 2009), *Oryza sativa* (Imin *et al.*, 2004; Lee *et al.*, 2009), *Cichorium intybus* (Degand *et al.*, 2009), *Festuca pratensis* (Chen *et al.*, 2012), *Pisum sativum* (Taylor *et al.*, 2005; Dumont *et al.*, 2011) as well as *Populus tremula* x *P. tremuloides* (Renaut *et al.*, 2004). Cold is known to significantly affect photosynthesis. Chen *et al.*, (2012) reported that two genotypes of meadow fescue (*Festuca pratensis*) differ in their frost tolerance with significant differences observed in protein abundance in these two genotypes exposed to cold. The differences were observed in the thylakoid-membrane-associated photosynthetic apparatus such as light-harvesting complexes, oxygen evolving complex, oxygen-evolving enhancer protein 1 (OEE1) and cytochrome b6/f complex iron sulphur subunit. An up-regulation of RuBiSCO subunits was reported due to cold stress (Gao *et al.*, 2009, Hashimoto and Komatsu, 2007).

1.3.1.4) Salt/Osmotic

Salinity is a constraint to crop production and other agricultural practices as it serves as one of the major environmental stresses. This type of stress has devastating global effects on arable land and it has been predicted that up to 50% of land will be lost by the mid-21st century (Wang *et al.*, 2003). High salt concentrations can also alter the texture of soil resulting in decreased porosity and consequently reduced soil aeration and water conductance. There has also been cross-talk between high salt stress and drought stress. Increased soil salinity generates a low water potential zone, making it even more difficult for the plant to obtain water and the necessary nutrients for survival (Mahajan and Tuteja, 2005). Therefore, osmotic stress results in water shortage conditions in the plant and takes the form of physiological drought. The major ions in salt stress signalling are Na^+(toxic to cell metabolism and adversely affects some enzymes activity), K^+ (required for maintaining osmotic balance, stomatal conductance and a co-factor for many enzymes such as pyruvate kinase) H^+ and Ca^{2+}(reduces the toxic effects of NaCl by initiating the stress signal transduction leading to salt adaptation) (Mahajan and Tuteja, 2005; Niu *et al.*, 1995), which brings homeostasis in the cell.

1.3.1.5) Light

Plants produce their own energy and metabolites through photosynthesis. Although photosynthesis requires sunlight, it also has a negative effect on photosynthesis. Plants have therefore evolved protective and response mechanisms to mitigate the negative effects of light stress. Excess light can be generated not only by intense light but also by a variety of other factors which limit the capacity of the photosynthetic machinery. Among such factors are water and nutrient shortages as well as high levels of pollutants. By slowing down the rate of photosynthesis, these factors reduce the threshold of light intensity perceived as excessive by the photosynthetic machinery. Plants activate several lines of defense to avoid irreversible damage due to light stress. The understanding of such photo-protective mechanisms is critical in view of the effects of light stress on plant productivity (Demmig-Adams, 1987; Demmig-Adams and Adams, 1992; Horton *et al.*, 1994).

Various plants vary in their ability to tolerate light stress. These differences sometimes reflect the use of different defense or adaptation strategies. Photosynthetic organisms displaying extreme light-stress tolerance offer interesting objects for investigating unique, or widely conserved photo-protective mechanisms (Demmig-Adams, 1987; Demmig-Adams and Adams, 1992).

1.3.1.6) pH of Soil

The acidity or alkalinity of soil is expressed as the total hydrogen ion concentration in the soil water solution. Neutral soil is represented by a pH of 7, values 7 to 0 are referred to as acidic and 7 to 14 are increasingly alkaline. In practice, the pH of soil ranges from 3 to 9, with the majority between the range of 4.5 and 7.5 (Traina *et al.*, 1985). Most soil used for agricultural practices become gradually more acidic as calcium is lost due to leaching by rainwater and irrigation. The process is further accelerated by the use of nitrogenous fertilisers such as ammonium nitrate and ammonium sulphate. Vegetables and other crops vary in their tolerance to soil acidity. Acidic soil often causes yellowing of leaves, stunted growth and affects the yield of crops as the pH level falls. Furthermore, plants grown in adverse pH conditions may be more prone to disease and fungal attack (Baath *et al.*, 1980). The availability of plant nutrients is considerably affected by soil pH. Calcium, potassium, magnesium and sodium are alkaline elements, which are lost with increasing acidity whereas phosphorous is more available in acidic soil conditions. Acidity can also induce deficiencies of micronutrients such as molybdenum, copper and boron, although a deficiency in the latter is more commonly seen in alkaline soils where over-liming has occurred. Other minor elements which may be deficient due to low solubility in high pH includes manganese and iron, the deficiency of which produces a chlorotic condition commonly seen in the young leaves of brassica plants (Barrow, 1984).

1.3.1.7) Summary

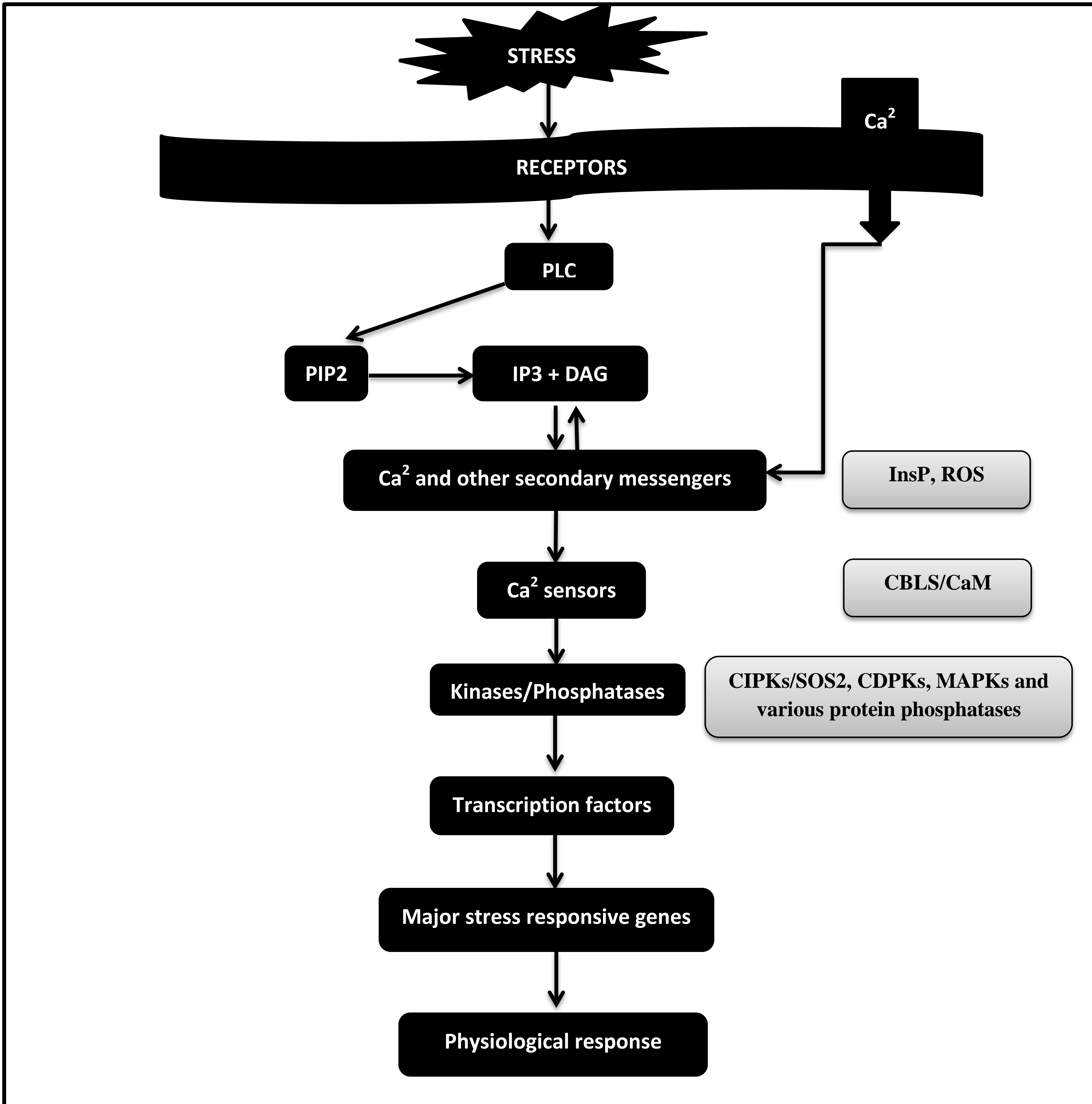


Figure 1.1: A general overview of an abiotic stress signalling pathway. Adapted from Mahajan and Tuteja (2005).

Various studies have reported that there are transcription factors or rather various genes that are differentially regulated to variations in the plant proteome which can mitigate the effect of the various stresses through the development of stress tolerance and resistance (Abe *et al.*, 1997; Sakamoto *et al.*, 2004; Kizis *et al.*, 2004; Lu *et al.*, 2007; Xiang *et al.*, 2008; Wang *et al.*, 2009). Other factors reported during abiotic stress for example include temperature regulation of genes such as *Td6Tm1* that codes for receptor proteins found to be involved in plant defense and adaption responses in Triticum aestivum (Lehti-Shiu *et al.*, 2009).

In summary (Figure 1.1): the stress is first perceived by receptors situated in the plant's cell walls or membranes. A signal is then transduced downstream, eliciting second messengers including calcium, ROS and inositol phosphates. Intracellular calcium levels become further modulated by inositol phosphates. Calcium binding proteins senses thus increase in calcium levels and alter their conformation in a calcium dependent manner, interacting with their respective partners and thus initiate a phosphorylation cascade. This then targets the major stress responsive genes or the transcription factors controlling various genes. The products of these stress genes ultimately lead to plant adaptation and aid in the plant's survival. Consequently, plants respond to stresses as individual cells and synergistically as a whole organism. The generation of abscisic acid (ABA), salicylic acid (SA) and ethylene also comes into play during stress induced changes in gene expression. These molecules both amplify the initial signal and initiate a second round of signalling that may follow the same pathway or use altogether different components of other signalling pathways (Mahajan and Tuteja, 2005).

1.3.2) Biotic Stress Responses

1.3.2.1) Introduction

Biotic stress factors on the other hand are usually a result of pathogens such as viruses, bacteria and fungi, herbivores or insects. Resistance to biotic stress may either be qualitative or quantitative. Biotic stress is a consequence of the parasitic relationship where the pathogen, herbivore or insect derives food from its host. The parasitic organism can either live and multiply in another living system known as biotrophic or live, feed and multiply on dead tissue known as necrotrophic. Plants have developed different defense mechanisms to protect themselves from biotic stresses.

These lines of different defense mechanisms often overlap with one another (Valcu *et al.*, 2009). The stress is first perceived by the receptors present on the membrane of the plant cells.

1.3.2.2) Stress Response to Pathogens

Plants have developed defense mechanisms that protect them from various pathogens (Kano *et al.*, 2011). A plant's successful defense against pathogen attack depends on the prompt recognition of the pathogen's attack and up-regulation of appropriate defense mechanisms (Ebel and Cosio, 1994; Jones and Takemoto, 2004) such as phytoalexins, reinforcement of plant cell walls, ROS production as part of the hypersensitive response, pathogenesis-related (PR) proteins and others possessing anti-microbial properties (Bailey *et al.*, 1976, Alvarez *et al.*, 1998; Mittler *et al.*, 2004; Nicholson and Hammerschmidt, 1992; Van Loon and Van Strien, 1999). Pathogens produce pathogen-associated molecular patterns (PAMP's) that are perceived by plant receptors known as pattern recognition receptors (PRR), leading to PAMP triggered immunity. To overcome this response, pathogens produce effector proteins which then enter into the host cell. The host plant in response produces effector specific R-proteins which are encoded by R-genes to resist pathogen invasion. This gives rise to what is known as the hypersensitive reaction or effector triggered immunity and thus constitutes qualitative resistance. Further attack by the pathogen continues, producing enzymes and toxins. Several other signal transduction pathways, induction of phytohormones are also activated deploying broad biochemical resistance mechanism in order to minimize or suppress pathogen progress, thus constituting quantitative resistance (Kushalappa and Gunnaiah, 2013).

Plants use their cell wall to prevent fungal invasion through the deposition of a sugar polymer called callose. Callose is a β-1,3-glucan, first discovered in the mid 1800's and was described as a soft carbohydrate plug found in intercellular connections (Currie, 1957) predominantly in the area surrounding the site of pathogen attack (Kudlicka and Brown, 1997). Callose synthesis is initiated when β-1, 3 glucan synthase is activated subsequent to the disruption of plasma membrane integrity (Kauss, 1985). Formation of this soft carbohydrate plug occurs quite rapidly, mostly within minutes of wound initiation (Radford *et al.*, 1998; Nakashima *et al.*, 2003).

When viewed in electron micrographs, it appears electrolucent (Stone and Clarke, 1992) and fluoresces under UV light when stained with aniline blue fluorochrome (Stone *et al.,* 1985). Initiation of callose formation in response to wounding of plant cells is a result of a mechanism involving calmodulin (elevated Ca^{2+} ion levels) (Botha and Cross, 2001). Callose is largely produced in the sieve elements of the phloem to restrict sap through cells (Currier, 1957) and thus seal the damaged pores to prevent any further assimilate loss (Sjőlund, 1997). However, certain fungi have managed to overcome this defense mechanism by possessing highly developed haustoria that avoid activation of plant responses. This is accomplished by the encapsulation of haustorial complexes by callose and proposed to be a mechanism used by plants to accommodate certain beneficial types of fungi such as mycorrhiza (Maor and Shirazu, 2005). Once fungal pathogens manage to evade the first line of defense and enter the plant cell wall, a phytoalexin response is triggered. These molecules such as camalexins, saponins etc. are produced in response to signals indicating pathogen presence and subsequent infection (Thomma *et al.*, 1999; Yang *et al.*, 2004). These preformed inhibitors are glycosylated compounds that possess potential antifungal activity due to their role in defense against pathogenic microbes (Hammond-Kosack and Jones 2000; Osbourn, 2003).

1.3.2.3) Stress Responses to Insect Herbivory

Research has shown that insect herbivory is a significant limiting factor in food production with an estimated loss of up to 20% of crops per annum (Ferry, Edwards, Gatehouse and Gatehouse, 2004). This type of herbivory not only results in mechanical damage but may also result in pathogen transfer to affected plants (Ferry *et al.*, 2004). Unlike pathogen-induced responses, plants responses to insect herbivory are quite dynamic due to the evolution of insect defenses against plant protective systems. This interaction between plants and insects has generated a keen interest amongst scientists and has thus incorporated many fields such as physiology, ecology and biochemistry to mention a few. Plants undergo various chemical and morphological changes in their response to herbivory or pathogen attack (Karban and Baldwin, 1997).

These responses may either be constitutive or induced, involving various pathways which the affected plant activates in response to a specific insect, resulting in the differential activation of various signalling molecules (Walling, 2000). Insects feeding on plants may either result in a physical or a biochemical defense from the plant. Chewing insects induce a wound response by the plant, resulting in the production of anti-feedants such as alkaloids and protease inhibitors (Dangle and Jones, 2001). This wound response pathway is usually mediated by the oxylipin, Jasmonic Acid (JA) and ethylene which are also thought to act synergistically, stimulating their own respective biosynthesis in the process (Xu *et al.*, 1994; O'Donnell *et al.*, 1996).

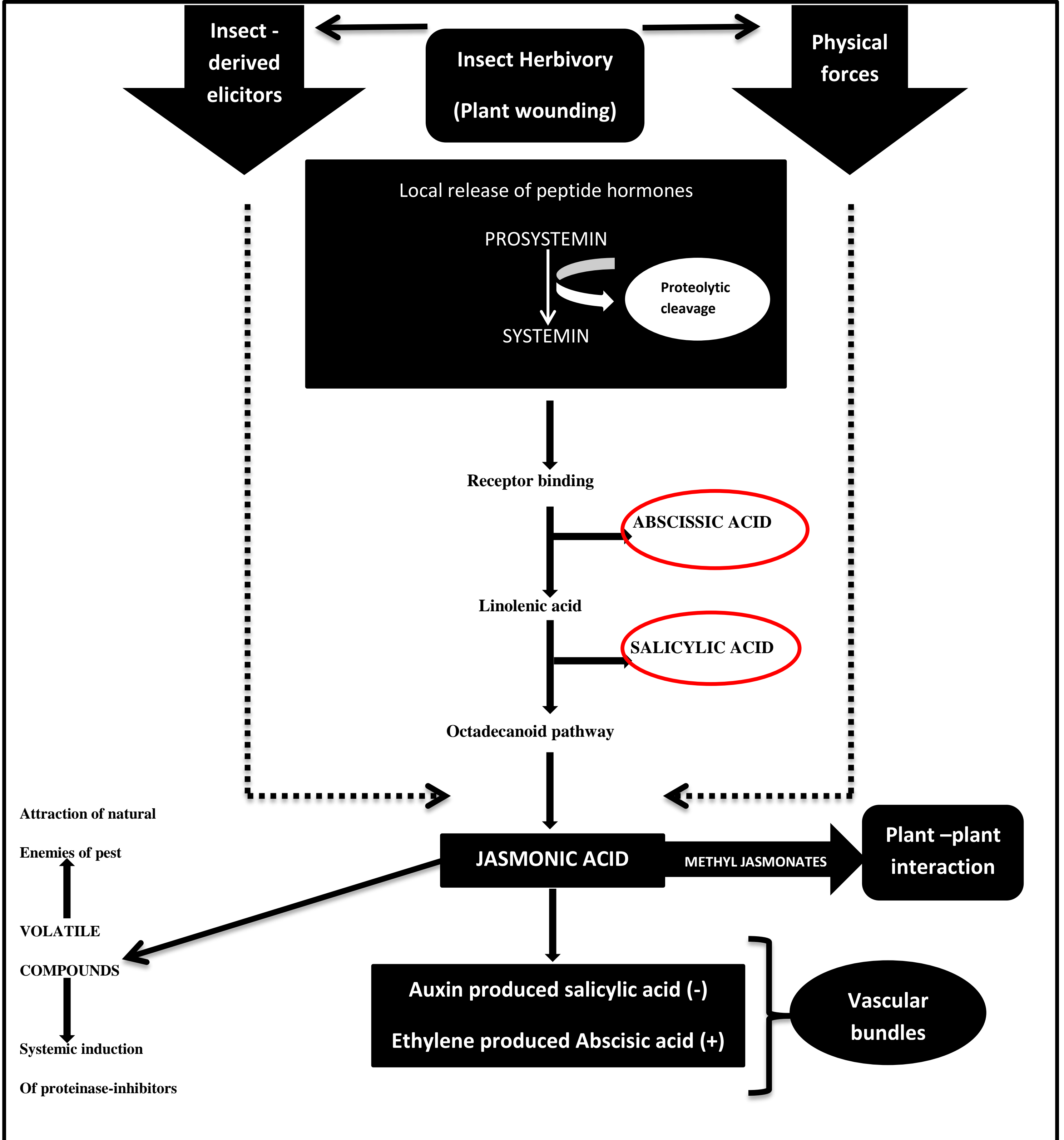


Figure 1.2: Wound response pathway initiated upon insect herbivory. Figure adapted from Ferry et al. (2004).

1.3.2.4) Stress Response to Phloem Feeding Insects

Various approaches have been used to obtain a better understanding of the stress response mechanisms induced by phloem feeding insects. Aphids are known as phloem feeders and cause severe plant loss worldwide (Powell *et al.*, 2006). Current literature indicates that plant responses to aphid feeding are more similar to that induced by bacterial infection as opposed to regular responses to insect herbivory (Botha *et al.*, 1996). They are highly efficient in colonizing and settling on plants. Secondly, winged adults colonize new host plants whereas wingless adults invest more resources in reproduction and tend to probe the plant for much longer periods (Powell *et al.*, 2006). Phloem feeding insects such as aphids probe a plant for longer periods, inducing leaf rolling for protection. Their survival is dependent on their ability to access phloem bundles while evading the plant defense responses (Tjallingii, 2006). Upon prolonged feeding on the sieve tubes, aphids continuously inject salivary secretions into the plant tissues with their stylets, which are thought to contain substances to counteract plant defense. Phloem feeders are capable of causing morphological changes, modified resource allocations and various local as well as systemic symptoms changes within the host plant (Goggin, 2007). Reports show that plant responses to phytopathogenous insects or pathogen attack share common events, for example protein phosphorylation, membrane depolarization, calcium influx and release of ROS such as hydrogen peroxide (H_2O_2). These events activate phytohormone-dependent pathways such as ethylene (ET) and jasmonate (JA) dependent responses; these being activated normally by necrotrophic pathogens (Thompson and Goggin, 2006) and grazing insects (Maffei *et al.*, 2007). Salicylic acid (SA) dependent responses are triggered by biotropic pathogens (Thompson and Goggin, 2006). Plants are able to differentially produce SA, JA and ET signalling molecules to adjust their response to the type of eliciting agent (De Vos *et al.*, 2005; Dicke *et al.*, 1999). Sucking insects such as aphids usually activate and are more associated with the salicylic acid pathway (Chen *et al.*, 2012). Phytohormone accumulation activates both local and systematic plant responses, thus leading to production and accumulation of defense proteins and secondary metabolites with antixenotic or antibiotic properties in damaged and undamaged parts of the plant. When it comes to plant aphid compatible interactions, a plant's SA-dependent response is activated, while expression of JA-dependent genes appears repressed (Walling, 2008; Thompson & Goggin, 2006).

1.3.2.5) Signalling Pathways Induced By Aphids

Aphid feeding induces various defense signalling mechanisms in plants. These mechanisms are thought to be the initial response of plants to reduce aphid attack. Smith and Boyko (2007) suggested that two different plant defense processes occur once an elicitor is detected by a plant. One process involves gene-for-gene recognition while the other process involves the activation of various signalling pathways which trigger a defense response (Morkunas *et al.*, 2011). Gene-for-gene recognition are thought to only occur in resistant plants, followed by the activation of aphid resistance and defense responses. The activation of signalling pathways in response to aphid attack alters gene expression which in turn alters cell molecular composition. On analysis of their *DN*A sequencing data, Zhu-Salzman *et al.* (2004) found that the encoded proteins function in photosynthesis, cell maintenance, defense signalling as well as in secondary metabolism. The recognition of aphids feeing by plant receptors are subsequently followed by the transmission of a cascade of defense events downstream that involves various signalling molecules. These plant signalling events are often driven by phytohormones such as Jasmonic acid (JA), Salicylic acid (SA), Ethylene, Abscisic acid (ABA), Gibberellic acid (GA), Auxins (IAA) and free radicals (ROS) mainly Hydrogen peroxide (H_2O_2) and Nitric oxide (NO) (Gao *et al.*, 2007). These systemic signalling pathways in plants can mount a multiple layered defense throughout the entire plant system and may result in leaf or phloem modification, plant cytoskeletal modification, production of secondary metabolites as well as transformed signalling to neighbouring plants (Mauch-Mani and Metraux, 1998).

1.3.2.5.1) Jasmonic Acid Induced By Aphid feeding

Jasmonic acid (JA) and its methyl esters come from a group of compounds known as oxylipins that are produced via oxygenation of fatty acids. JA and its related compounds, are involved in the regulation of plant responses to wounding and necrotrophic pathogens which have been described in Arabidopsis, tobacco, wheat and sorghum (Devoto and Turner, 2005).

The JA biosynthesis pathway together with lipoxygenase (LOX) being involved in it is important in wounding-induced defense responses and has been validated by the exogenous application of JA. LOX, the key enzyme has been studied in many species of plants in response to aphid feeding. The defense function of JA was first reported by Farmer and Ryan (1992), in which they provided evidence between wounding caused by insects, JA formation and protease inhibitor genes that prevent insect feeding. Furthermore, they proposed that wounding causes the formation of linoleic acid, which is the alleged precursor of JA. Korth and Thompson (2006) further provided evidence that JA and MeJA are potent inducers of protease inhibitors (PI) and play a pivotal role in the plants response to herbivore attack. Monaghan *et al.* (2009) reported that not only does JA and ethylene (ET) play key roles in resistance to herbivore attack but also to necrotrophic pathogens. Recently, it has been discovered that WRKY transcription factors are key elements of defense in the JA signalling pathway. A study conducted by Kusnierczyk *et al.* (2008) revealed that WRKY transcription factors play defense roles in *Arabidopsis* to the cabbage aphid (*Brevicoryne brassicae*). Cev1 (constitutive expresser of vegetative storage protein 1) alongside VSP (vegetative storage protein), PDF (plant defensin), Thi2.1 (thionin 2.1 and chitinase B have also been found to display constitutive activation of defense responses against various aphids (see Figure 1.3 (Ellis *et al.*, 2002).

1.3.2.5.2) Salicylic Acid in Plants Response to Aphid feeding

Salicylic acid (SA), a phenolic phytohormone has been found to regulate plant defense mechanisms against both abiotic and biotic stresses. It's involvement in systemic acquired resistance (SAR) is essential which is seen as a broad spectrum plant immune response and is also crucial for localized plant tissue hypersensitive responses (Cao *et al.*, 1994; Dempsey *et al.*, 1999; Vlot *et al.*, 2009; Vicente and Plasencia, 2011). SA is a central regulator of cell fate involved in gene expression, which is a process that engages the activation of plasma membrane bound NADPH oxidases. NADPH oxidases and cell wall peroxidases are responsible for the oxidative burst that takes place in the apoplast in response to the perception of abiotic and biotic stresses (Kawano & Muto, 2000).

The apoplastic oxidative burst and accumulation of resultant ROS – causing significant changes in cellular redox levels- in the extracellular space is characteristic of plant cells that are exposed to physical and chemical shock, insects and herbivores, symbiotic microorganisms and pathogens. NADPH oxidase activation serves to restrain the spread of pathogen- and SA-induced cell death (Pogány *et al.*, 2009).

SA has long been associated with resistance against biotrophic pathogens, in turn, stimulating the expression of defense response genes (PR genes) (Moran and Thompson, 2001; Monaghan *et al.*, 2009). It's involvement in response to aphid feeding has been observed in *Triticum aestivum, Arabidopsis thaliana* and barley indicating a possible resistance mechanism role. Mohase and van der Westhuizen (2002) have reported higher catalase and peroxidase (markers of resistance response) activities in resistant *T. aestivum* lines during SA induction. Their findings also showed that aphid infestation also inhibited catalase activity in both the resistant and susceptible plants, which probably owes to SA binding and inhibiting catalase activity. Kusnierczyk *et al.* (2008) also revealed that a wide range of defense response genes which are up-regulated due to aphid attack is dependent on SA. These include genes involved in SA synthesis (EDS1, EDS5 and PAD4) and stress responsive genes (PR1, PR2, PR4, PR5, NIMIN-1, NIMIN-2 and SABP2-like). Sorghum, for example, defends itself from the greenbug aphid (*Schizaphis graminum*) by up-regulating the expression of PR genes such as BGL2, HEL (hevein-like protein) and chitinases which are associated with the SA defense signalling pathway (Zhu-Salzman *et al.*, 2004). However, it is worth noting that there are certain SA-defense-related genes such as PR genes which are down-regulated during aphid infestation. This was reported by Park *et al.* (2006) who identified genes in sorghum using c*DN*A subtraction and Microarray analysis in response to greenbug phloem-feeding.

1.3.2.5.3) Ethylene in Plants Response to Aphid feeding

Ethylene (ET), a gaseous plant hormone is synthesized in response to stress or pathogen attack, but also affects a myriad of developmental processes, plant germination, leaf abscission, root nodulation and programmed cell death (Johnson and Ecker, 1998). ET is usually perceived by a group of membranous receptors, ETR1/ETR2 and ethylene response sensor 1 (ERS1/ERS2) (Chang and Stadler, 2001).

Biochemical and genetic studies revealed that these receptors are negative regulators of ethylene response and are inactivated by ethylene binding (Guo and Ecker, 2004). On the other hand ethylene also has positive regulators that are acting downstream of CTR1 such as EIN2, EIN3, EIN5, and EIN6. Studies have showed that ethylene can lead to the activation of *Arabidopsis* 40-kDa proteins that have MAPK characteristic properties (Guo *et al.*, 2001). Reports on aphid infestation on alfalfa, barley and wheat show that higher ET concentrations were observed in susceptible rather than resistant cultivars. However, several factors such as plant cultivar, plant age, degree of infestation, type of aphid and length of infestation come into play (Dillwith *et al.*, 1991; Anderson and Peters, 1994; Miller *et al.*, 1994). In Arabidopsis, Moran *et al.* (2002) found activation of genes in response to *M. persicae* feeding. These genes function in oxidative stress, PR genes and calcium dependent signalling. Also, during this activation period, induction of 1-aminocyclopropane-1-carboxylic acid (ACC), a gene encoding an ethylene biosynthesis gene was noted. In a different study, Divol *et al.* (2005) observed that in aphid susceptible celery, ET-responsive EREBP-3 and ET responsive elements are up-regulated by *M. persicae*.

1.3.2.5.4) Abscisic Acid (ABA) and Gibberellic Acid (GA) in Plants Response to Aphid feeding

Abscisic acid (ABA) is a positive regulator of leaf senescence that accumulates in response to drought, salt or extreme temperatures, and leads to altered gene expression and adaptive responses such as stomatal closure and the build-up of osmo-compatible solutes (Chandrasekhar *et al.*, 2000). Studies conducted by Jammes *et al.* (2009) and Xiong and Yang (2003) indicated that ABA synthesis and MAPKs signalling cascades play vital roles in stomatal movement under drought stress. Studies by Robert-Seilaniantz *et al.* (2007) reported interesting and important roles for both ABA and GA in plant defense against biotic stress. The role of ABA has been implicated in basal and induced resistance against biotic stress (Flors *et al.*, 2009). Miller *et al.* (1994) reported that ABA and GA have no effects on aphid feeding, while Flors *et al.* (2009) showed that ABA plays an important role during plant-insect interactions. Park *et al.* (2006) as well as Boyko *et al.* (2006) further investigated and reported the up-regulation of genes such as ASR (ABA-water stress-ripening-induced protein) in the biosynthesis of ABA.

Furthermore, the GA-induced protein (GIP) and the seed maturation protein were differentially regulated by greenbug feeding. In addition, Zhu-Salzman *et al.* (2004) reported that the drought, salt and low temperature responsive gene (DRT) as well as the aldehyde oxidase gene (AOX) which are known to be regulated by ABA, were reported in response to greenbugs.

Previous studies have shown that GA serves as a plasma membrane-bound receptor and has been implicated in plants' defense response by regulating β-1, 3-glucanase from cereal grain (Matsuoka, 2003).

1.3.2.6) ROS and NO involvement in plant response to aphid feeding

Plants naturally produce Reactive oxygen species (ROS) as signalling molecules to control various processes including pathogen defense, 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). However, H_2O_2 is relatively stable ROS. The production of H_2O_2 is a general response of plants to general stress conditions and not necessarily specific to plants infested with aphids (Wojtaszek, 1997). The H_2O_2 activates defense genes such as peroxidases and higher levels could have toxic effects on aphids, causing damage. Reports by Kusnierczyk *et al.* (2008) have shown ROS involvement in early signalling in *Arabidopsis thaliana* after infestation by aphids, *Brevicoryne brassicae*. H_2O_2 has also been found to activate the protein phosphorylation cascade, which modulates gene expression in response to external stimuli. These cascades of events also involve the phosphorylation of events of MAPK. Parker *et al.* (2006) reported the overall down-regulation in resistant and susceptible sorghum lines of the CAT, POX and QR genes using microarray analyses. This overall down-regulation is thought to be due to intense ROS burst during the early stages of the greenbug aphid feeding. Therefore the levels of ROS remained significantly high to induce defense related genes. Several mechanisms have also been reported to be associated with the production of ROS in response to infection with NADPH oxidase being 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). Thus, 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).

Nitric oxide (NO), together with ROS is recognised to increase levels after pathogen attack. These molecules have been found to activate the plants' hypersensitive response, stimulate SA production as well as induce defense response genes (Walling, 2008). Moloi and van der Westhuizen (2005) reported NO's involvement in plant-aphid interaction as a defense response against the Russian wheat aphid. In their study, *D. noxia* induced early production of NO in two wheat isolines with higher levels reported in the resistant cultivar than in the susceptible cultivar. They have also reported that NO activates SA biosynthesis as well as interacts with ROS radicals. It still remains unclear whether NO could be a signal molecule in the resistant responses of wheat to *D. noxia*.

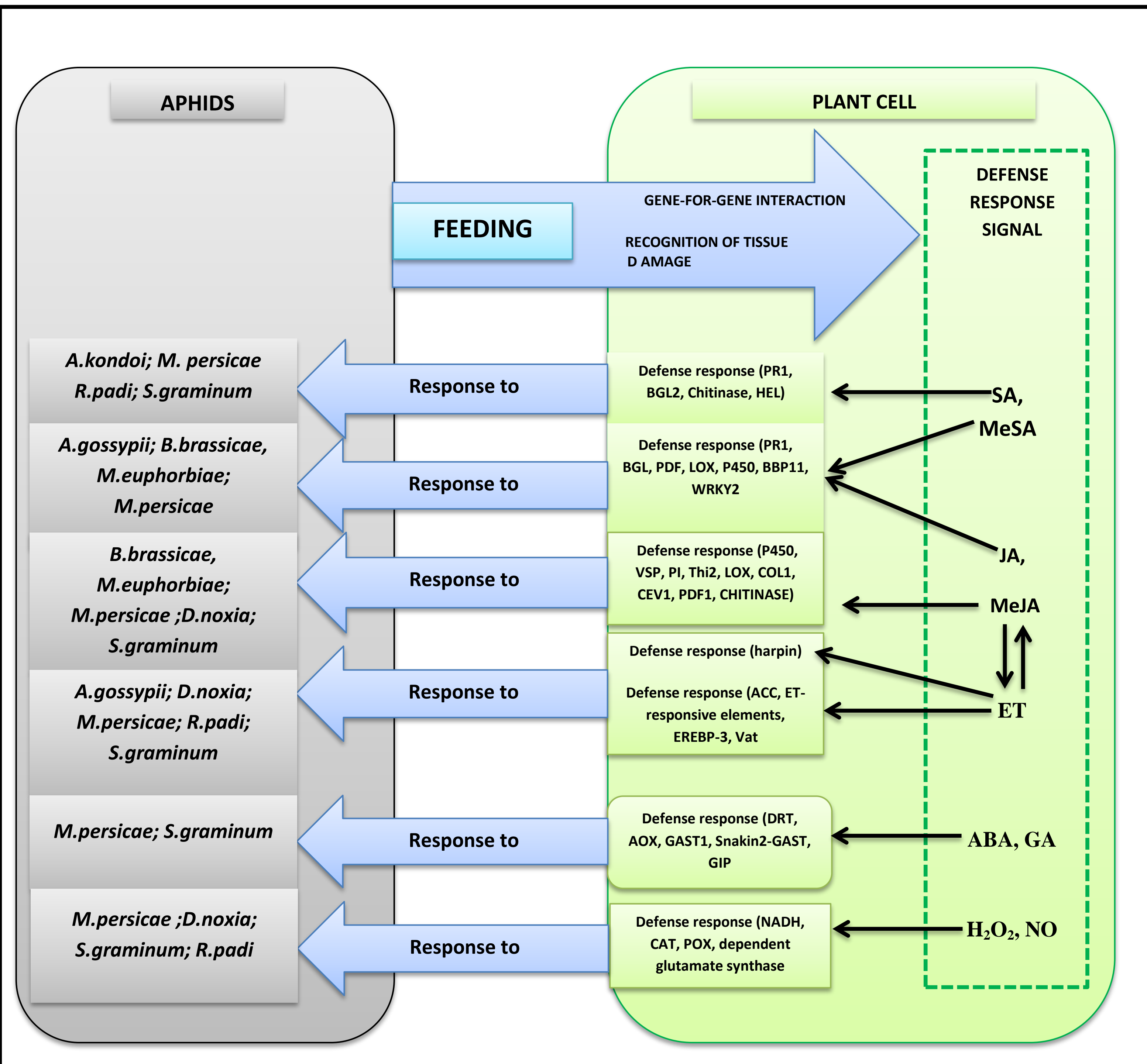


Figure 1.3: Schematic representation of plant signalling pathways involving defense responses to aphid feeding. Adapted from Morkunas *et al.* (2011).

1.3.3) Resistance responses in plant

Plants are in a continual arms race and constantly being challenged by herbivores and microorganisms. Plants are sessile and therefore lack systems which will permit them to shield themselves in a manner similar to that of animals (Botha *et al.*, 2005). For these reasons, they have developed chemical and physical defense mechanisms to protect themselves against attack (Slater *et al.*, 2004). External defenses that the plants have in place to discourage feeding includes thorns, cuticle, bark or wax layers. Once a pest has successfully penetrated these external defenses, it encounters a further array of internal defense responses which may include structural, chemical or proteins/ enzymes defenses (Chisholm *et al.*, 2006). Many proteins, enzymes, defensins and protease inhibitors are employed to inhibit pathogens and inhibit digestion within the pest. Chitinases, glucanases and other lysosomal enzymes are produced in reaction to infections and infestations (Freeman and Beattie, 2008).

The mechanisms essential to plant-aphids interactions still remains a field that needs to be explored. Nevertheless, plants do display adaptations that limit aphid infestation. Defenses that are shared by all genotypes of a plant species and that prevent a species from being a host for a particular pest constitute non-host resistance. Traits that reduce herbivores from settling or colonizing (antixenosis) or reduce herbivore survival and production (antibiosis) on a host species are sources of host plant resistance. A third type of resistant mechanism that occurs is tolerance which occurs when the plant is able to tolerate the nutrient drain caused by the aphids (Smith and Boyko, 2007).

Most aphids have been found to inflict minimal damage as opposed to other herbivores (Walling, 2008). They use their stylets to penetrate the plant, enabling them to feed for sustained periods so as to obtain enough nutrients while mimicking damage to the plant. Several studies have indicated that plants respond to RWA feeding is a typical Hypersensitive Response (HR) that includes the induction of peroxidases, β-1, 3-glucanases and chitinases. It has been proposed that the resistance response of wheat to aphid feeding is elicited mainly in the apoplast where several defense related products accumulate (Bowles, 1990). Van der Westhuizen et al. (1998) found that RWA feeding on resistant wheat cultivars gave rise to increased levels of β-1, 3-glucanase. In an analogous study, noticeably higher amounts of β-1, 3-glucanases and chitinases were reported in resistant Tugela Dn as opposed to the susceptible Tugela cultivar (Botha *et al.*, 1995, Van der Westhuizen and Pretorius, 1996).

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 defense enzymes/small molecules (Harris *et al.*, 2003).

The Systemic Acquire Resistance (SAR) response follows the initial and rapid HR, which results in a broad-spectrum, long-lasting protection in non-infected tissues in certain plants that have suffered infections due to pathogen attack (Hunt *et al.*, 1996). The onset of SAR is usually characterized by the presence of Salicylic Acid (SA) and hydrogen peroxide (H_2O_2) molecules, giving rise to pathogenesis related (PR) genes which usually spread throughout the rest of the plant (Malamy *et al.*, 1990; van Loun, 1985; van Loun and Antoniw, 1982). 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).

1.3.4) Resistance genes and resistance mechanism

Plants are continuously under threat by a variety of insects and pathogens and have thus needed to develop defense approaches which are regulated by phytohormones to ensure survival (Jankielsohn, 2013; Lazebink *et al.*, 2014). This has resulted in the evolution of

insect biotypes which have evolved particular resistance (Sugio *et al.*, 2014). Since the new biotypes exert a selection pressure on the host plant, those plants which have developed a new defense strategy will survive thereby resulting in the development of new resistance in the plant (Sugio *et al.*, 2014; Bruce, 2015). 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 requiring the constant development of new resistant cultivars. RWA resistant cultivars have been bred throughout the world and therefore numerous commercially resistant cultivars are accessible 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 distribution of genetic resources for resistance also assists in achieving to produce stability without resorting to the use of harmful chemicals (pesticides and insecticides). Furthermore, it prevents the environment against 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 which is posed by various *Dn* genes may be categorised as antibiosis, antixenosis or tolerance, although some cultivars may display a combination of these (Smith *et al.*, 1992; Haile *et al.*, 1999). In antibiosis, the plant limits the aphid‘s rate of growth by affecting its biology thus reducing the fecundity rate of the insect and it is the *Dn*1 gene that is responsible (Wang *et al.*, 2004; Botha *et al.*, 2014). In antixenosis, the resistant cultivar prevents 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).

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 the genes associated with RWA resistance (Adapted from Van Zyl, 2007).

Gene	Resistance Source	Mode of Inheritance	Reference
Dn1	PI127739	SD	Du Toit, 1989
Dn2	PI262660	SD	Du Toit, 1989
Dn3	*T.tauschii* line SQ24	R	Nkongolo, 1991
Dn4	PI372129	SD	Nkongolo, 1991
Dn5	PI294994	SD	Marais and Du Toit, 1993
Dn6	PI243781	SD	Dong and Quick, 1995
Dn7	94M370	SD	Marais *et al.*, 1994
Dn8	PI294994	SD	Liu *et al.*, 2001
Dn9	PI294994	SD	Liu *et al.*, 2001
Dnx	PI220127	SD	Liu *et al.*, 2001
Dny	PI220350	Unknown	Tolmay *et al.*, 2012

SD: Single dominant; CD: Co-dominant; R: Recessive

These genes were introduced into lines with acceptable agronomical traits through backcross breeding. The donor parent is crossed with the recurrent parent and the progeny is further crossed with the recurrent parent to establish a line identical to the recurrent parent with the addition of the gene of interest (Dogimont *et al.*, 2010). Backcross breeding was performed to transfer resistance into well adapted South African cultivars of Tugela, Betta, Molopo, Karee, Kariega, Letaba, Molen and Palmiet at the Small Grain Institute in Bethlehem, South Africa (Tolmay *et al.*, 2006). During the backcross breeding process, plants were screened for resistance to Russian Wheat Aphid.

1.3.5) Photosynthesis

Photosynthesis plays a major role and is a fundamental process in the plant kingdom that ensures survival of plants. The conversion of light energy into chemical energy is key to the survival of plants. The photosynthetic reaction systems, which play a pivotal role in the energy conversion process, are composed of special protein-chlorophyll complexes in the core of light-harvesting photosystems (Buttner *et al.*, 1992). Photosynthesis takes place as a major energy harvesting reaction which involves two photosystems: the oxygen-evolving

photosystem II (PSII) that originated from purple bacteria and the ferredoxin reducing photosystem II (PSI) that originated from the green sulphur bacteria (Buttner *et al.*, 1992). Chlorophyll a and Chlorophyll b which are the primary pigments are located in the thylakoid membrane. These pigments absorb different light and accumulate the energy in excited electrons in the thylakoid membrane. Secondary pigments, carotenoids (Carotenes and Xanthophyll) are located in the chloroplast membrane and outer membrane to absorb the light waves not efficiently absorbed by chlorophyll (Nelson and Yocum, 2006).

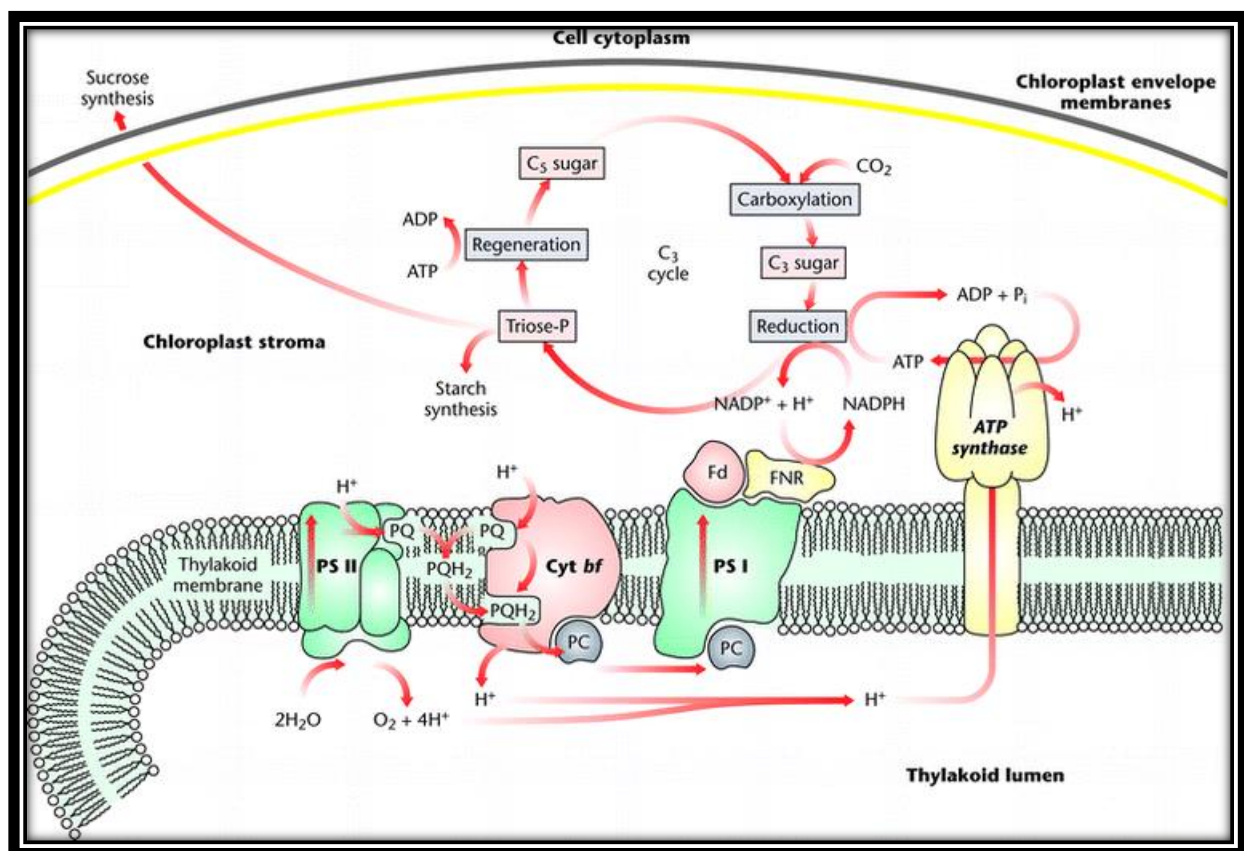


Figure 1.4: Photosystem I and II location. The thylakoid membrane with PSI and PSII are indicated with the movement of reactions to produce sugars through the Calvin cycle. Figure taken from www.ualr.edu/botany/botimages.html

In Photosystem II, the P680 reaction centre captures photons. This reaction centre complex consists of D-1 and D-2 polypeptides, five chlorophyll a, two pheophytin a, one B-carotene, and one or two cytochrome b-559 heme(s) (Nanba and Satoh, 1987). Light energy is used to carry out the splitting of water molecules. When the electrons are released from the water, the water molecule is broken into oxygen, which is released into the atmosphere, and hydrogen

ions are used to drive ATP synthesis. The electrons, excited at the antenna molecule P680, are passed down a chain of electron-transport proteins and receive extra electrons from PSI. More hydrogen ions are pumped across the membrane as these electrons flow down the chain providing more protons for ATP synthesis. The electrons are then transported on a NADPH molecule to enzymes that build sugar from water and carbon dioxide (Nelson and Yocum, 2006). A photosystem I reaction center complex consists of 6 polypeptides containing two of subunit I that associate with P700, subunit PSI-D, subunit PSI-E, quinones and fluorenones. Twenty chlorophyll a molecules and a cytochrome 522 heme form the complex P700 molecule (Bengis and Nelson, 1977).

Photosystem I also uses photons, but at 700 nm wavelength, to excite electrons from its antenna molecule P700. The electrons produced by PSI are captured by ferredoxin and used to reduce NADP+ to NADPH. ATP, which is produced via chemiosmosis, and NADPH are then used in the light-independent reactions, together with hydrogen atoms extracted from water by PSII, to convert carbon dioxide to glucose and in the process release oxygen as a by-product (Fromme, 1996; Nelson and Yocum, 2006).

Phloem-feeding insects (PFI) not only significantly reduce photosynthesis in their host plants (Macedo *et al.*, 2003) by down-regulating the expression of photosynthesis-related genes (Heidel and Baldwin, 2004; Voelckel *et al.*, 2004; Zhu-Salzman *et al.*, 2004; Qubbaj *et al.*, 2005; Yuan *et al.*, 2005), but also cause a changed carbohydrate metabolism by resource allocations from a growth to a defense metabolism (Heidel and Baldwin, 2004). The changes in metabolism are due to the pathogen that manipulates the carbohydrate metabolism of the plant for its own advantage. The plant, upon recognition of the aphid, also increases carbon supply to the areas of need such as defense reactions (Berger *et al.*, 2004). In resistant cultivars such as Tugela Dn, genes required for Rubisco synthesis are upregulated, which might be necessary for sustaining energy production for resistance (Van der Westhuizen and Botha, 1993). Source–sink relationships and water relations are also modified within the plant, because PFI must extract large volumes of phloem sap to attain adequate nitrogen (Douglas, 2006). By inducing genes involved in carbon assimilation and mobilization to increase sugar uptake, the sugars are depleted and thereby localized metabolic sinks are created (Moran and Thompson, 2001; Moran *et al.*, 2002; Zhu-Salzman *et al.*, 2004). PFI also modifies nitrogen allocation in their hosts by upregulating genes involved in nitrogen assimilation, in particular genes that encode enzymes required for synthesis of tryptophan and

other essential amino acids (Sandstrom *et al.*, 2000; Heidel and Baldwin, 2004; Zhu-Salzman *et al.*, 2004; Thompson and Goggin, 2006).

1.3.6) The effect of RWA feeding on photosynthesis

The photosynthetic machinery of a plant is significantly reduced by RWA feeding as the expression of genes involved in chloroplast synthesis and function is inhibited (Botha *et al.*, 2006). Photosynthesis-related genes are strongly suppressed by MeJA signaling, and to a lesser extent by SA (Zhu-Salzman *et al.*, 2004).

RWA feeding destroys the cell membranes, damages the chloroplasts, and thus, effective photosynthetic capacity declines (Fouché *et al.*, 1984). The decrease of total chlorophyll (a and b) and carotenoids (luteins), which play an important role in the PSI, were observed by Heng-Moss *et al.*, (2003) in susceptible cultivars after RWA feeding. This suggests that RWA feeding causes the reduction of the photosynthetic rate in susceptible lines and the PSII complex is suggested to be a target for RWA damage (Heng-Moss *et al.*, 2003). Chloroplast machinery maintenance is one of the determining factors in enabling resistant varieties to overcome the stress during RWA feeding (Botha *et al.*, 2006). Furthermore, Botha *et al.* (2006) obtained 200 non-redundant expressed sequence tags (ESTs) from the RWA resistant wheat transcriptome elucidated in response to RWA. Of these genes, 18.9% were involved in photosynthesis including chloroplast genes for the Rubisco rbcL, red chlorophyll catabolic reductase, the Photosystem I P700 apoprotein, thioredoxin m and chloroplast ATP synthase. Upon RWA feeding, ATP synthase and was found to be significantly higher expressed in resistant Tugela Dn when compared to the susceptible Tugela cultivar. It would therefore appear that this enzyme/protein is important for maintenance of photosynthetic activity in resistant wheat during RWA feeding. These genes thus serve as promising candidates to consider for expression manipulation to ultimately find a means of generating RWA resistant wheat lines (Both *et al.*, 2006).

1.3.7) Aphids

The family aphididae comprises more than 4300 species all of which are specialized to feed on phloem sap (Blackman and Eastop, 1994).The word 'aphids' is a broad term generally referring to any member of the aphididae though there are sometime uncertainties about the families to be included (Iharco and Van Harten, 1987). These species are characterized by different feeding behaviour (Blackman and Eastop, 1994). Aphids feed from the sieve elements in which they use specialized stylet-like mouthparts to probe intercellularly (Pollard, 1972) and produce large amounts of honeydew, sugary excreta. Majority of aphids that lie within the group aphididae, do little perceptible damage to plants (Miles, 1999). However, exceptions do exist though with the greenbug, *Schizaphis graminum* and the Russian wheat aphid, *Diuraphis noxia*.

These have been found to cause significant plant damage for two reasons (Dixon, 1998): firstly, due to their role as vectors of various pathogens that enter host plants' tissue while various affected aphid species feed on it and secondly because they secrete a proteinaceous salivary sheath that lines the stylets path as well as a watery based saliva containing enzymes such as peroxidases, β-glucosidases, oxidases, pectinases, cellulases and a number as of yet uncharacterized enzymes (Mathews, 1991; Miles, 1999). Aphids induce upregulation of PDF 1.2 and Lox 2 genes in plants they feed on. These genes encode for defensin (former) and lipoxygenase (latter) and are involved upon wounding in the JA signalling pathway (Walling, 2000). Their feeding has been reported to cause reduced or altered photosystems, decreased plant growth and decreased synthesis of many metabolites (Todd *et al.*, 1971). Studies have proposed that phloem feeding insects such as aphids trigger similar responses in plants to that of feeding insects despite the aphids probing action on the host plant. This is observed by the activation of JA and SA- mediated signalling pathways. Furthermore, plant responses to aphid feeding include the production of ROS, cellular accumulation of SA and subsequent expression of PR proteins (Moran *et al.*, 2002). Studies have also shown that aphids introduce foreign substances such as oligosaccharides and glycoproteins occurring in aphid saliva into their host plant (Moran and Thompson, 2001).

1.3.7.1) The Russian Wheat Aphid (RWA) (*Diuraphis noxia*)

Diuraphis noxia Kurdjumov, also generally known as the Russian Wheat Aphid is a small, yellow-green or green-grey phloem feeding insect that is approximately 1.4 - 2.3mm in length.

Figure 1.5: The Russian Wheat Aphid. Adapted from Tolmay, 2006. [Photograph: J.L. Hatting]

The insect appears to have a host preference that includes cereal grasses, favouring mostly wheat and barley. This aphid is endemic to central Asia, Southern Russia, countries bordering the Mediterranean Sea, Iran, Afghanistan and it has also been suggested that *D. noxia* coevolved with triticale in the Fertile Crescent. It then distributed from Western Asia to Africa and the USA. The first reports of *D. noxia* in South Africa was in 1978 and by the fall of 1979 it had become a serious pest, spreading throughout the wheat producing areas of the Western and Eastern Orange Free State as well as Lesotho (Walters *et al.*, 1980). By the mid 1990's it had successfully spread to all wheat producing countries except Australia (Basky, 2003) with most severe effects on wheat production in South Africa and the USA (Smith *et al.*, 1992; Tolmay *et al.*, 2007; Smith *et al.*, 2004). The estimated economic loss caused by this pest was found to be around $900 million in the USA between the period 1987 and 1993 (Morris and Pears, 1998) but further studies have shown that this figure had significantly increased.

This was due to the development of new *D. noxia* biotypes (Botha *et al.*, 2010) and was also reported to be the case by Tolmay *et al.* (2007) in South Africa. Several *D.noxia* biotypes exist in South Africa, namely RWA-SA1, RWA-SA2, RWA-SA3 (Jankielsohn, 2011), RWA-SA4 (Jankielsohn, 2014) and a laboratory developed strain which is highly virulent, SAM (Van Zyl *et al.*, 2005). The work developed in this dissertation focuses on understanding the resistance mechanism activated by the RWA resistance gene *Dn*1 during infestation with RWA-SA2 on Tugela Dn and whether RWA-SA2 overcomes the resistance response activated by the *Dn* gene. Although *D. noxia* has the ability to produce sexually and asexually, no male species have been identified in South Africa (Puterka *et al.*, 1993). The ability of a pest to infest a new host has also been found to be variable in magnitude, otherwise known as virulence. This variation in virulence was noted with pests from different geographical regions (Puterka *et al.*, 1992; Smith *et al.*, 1992; Burd *et al.*, 2006). Notable physical symptoms due to virulence by the pest include longitudinal streaking, stunted growth, leaf rolling and chlorosis. This further leads to reduced photosynthetic activity, while the phytotoxic saliva through aphid stylets causes the breakdown of cellular membranes and chloroplasts in susceptible plants (Bird and Elliott, 1996; Botha *et al.*, 2005). Not only does aphid saliva function in possible hydrolytic activity but also moistens food, facilitates mechanical penetration of cells during feeding, assist in dissolution of plant material as well as protects stylets and prolongs feeding time (Miles, 1959). Aphids contain two types of salivary glands, namely the primary and accessory glands. The primary glands are more differentiated than the accessory glands. Two types of salivation have been proposed by Tjallingii (2006): E1 and E2. E1 occurs when the aphid probes into the sieve elements and E2 once the aphid has settled after the phloem has been detected. This saliva is thought to contain phenolic compounds, amylases, pectinases, cellulases, lipases, amino acids and other proteolytic enzymes to mention a few (Miles, 1972; Fouchi *et al.*, 1984; Robinson, 1992; Ni and Quinsenberry, 2003).

1.3.7.2) Russian Wheat Aphid Biotypes

Several biotypes exist not only in the South African context but in Russian Wheat Aphid endemic areas as can be expected as selective pressure has forced the emergence of these biotypes which can overcome the resistance presented by the resistant cultivars.

The severity of attack of these biotypes varies markedly (see table 1.2 but the biotypes are quite similar in morphology. This is characterized by their feeding behaviour and classification was first initiated by Puterka *et al.* (1992).

Table 1.2: Summary of the South African RWA biotypes; year of identification and the genes they are virulent against.

Biotype	Year Identified	Virulent against	Reference
RWA-SA1	1978	*Dn3*	Jankielsohn, 2011
RWA-SA2	2005	*Dn1, Dn2, Dn3 and Dn9*	Jankielsohn, 2011
RWA-SA3	2009	*Dn1, Dn2, Dn3, Dn4 and Dn9*	Jankielsohn, 2011
RWA-SA4	2011	*Dn1, Dn2, Dn3, Dn4, Dn5 and Dn9*	Jankielsohn, 2014

As previously mentioned, several biotypes exist from a South African context with Russian Wheat Aphid biotype 2 (RWA-SA2) being the focus point of this study. This biotype appeared several years ago and appeared more virulent than RWA-SA1 on existing RWA-resistant wheat lines (Tolmay *et al.*, 2007). A study conducted by Jimoh *et al.* (2011) reported that RWA-SA2 breeds faster on resistant and non-resistant barley lines. Physiologically, one of the most readily apparent symptoms attributed to RWA infestation is that of callose deposition (Benhamou, 1992).

Callose, as mentioned previously is a linear β-(1, 3) glucan that constitutively occurs in cell walls of intact tissues such as sieve plates, cell plates of newly dividing cells, plasmodesmata and in reproductive organs during sporogenesis and gametogenesis as well as pollen tube development (Northcote *et al.*, 1989). It is rapidly synthesized and deposited in a localized manner in response to abiotic stress, wounding, mechanical stress and pathogen attack (Kauss, 1989; Bolwell, 1997; Benhamou, 1992). Wounding of plant cells and initiation of wound callose formation are said to be a result of elevated calcium ion levels in a mechanism involving calmodulin (Botha and Cross, 2001).

Studies conducted by Van der Westhuizen and Pretorius (1996) confirmed that intercellular proteins are expressed during RWA infestation on wheat and was particular the case in resistant cultivars. Their study also revealed that the difference in protein expression was mainly quantitative rather than qualitative. More recently, a study conducted by Nqumla (2012) in identifying specific signalling pathways involved in wheat resistance or stress response to aphid infestation found eight proteins that were identified as being up-regulated during aphid feeding (RWA-SA1) in wheat, and 11 proteins were identified as possibly involved in the wheat resistance mechanism against aphid infestation. Several proteins were also identified as constitutively expressed proteins, during normal conditions and aphid infestation. Most pathways identified with proteins up-regulated in the resistance mechanisms of Tugela Dn plants were related to energy metabolism and located in the chloroplast. Mudondo (2014) further investigated the effects of elevated carbon dioxide on RWA-SA1 resistance of Tugela Dn and found that the quantity of differentially regulated proteins were more under elevated carbon dioxide levels (450ppm) than at ambient conditions on analysis of her peptide profiles. This study aims to look at whether RWA-SA2 overcomes the resistance response activated by the *Dn* gene. This study will also further examine and contribute to existing knowledge in understanding the resistance mechanisms activated by the RWA resistance gene *Dn* (*Dn*1) by comparing responses following infestation of Tugela Dn with RWA-SA2 at ambient CO_2 levels. We also aim to look at whether RWA-SA2 has an impact on the photosynthetic activity of the plant. Several findings suggest that there is a large decrease in total chlorophyll (Fouché *et al.*, 1984; Burd and Burton 1992; Heng-Moss *et al.*, 2003; Wang *et al.*, 2004; Botha *et al.*, 2005), altered chlorophyll fluorescence induction kinetics, and reduced photochemical efficiency of photosystem II (Burd and Elliott 1996) have been noted with RWA feeding on susceptible hosts, but changes in leaf colour and a decrease in photosynthetic capacity have not been reported for resistant cereal hosts (Heng-Moss *et al.*, 2003; Wang *et al.*, 2004; Botha *et al.*, 2005). However, it still remains tentative what impact RWA-SA2 will have.

1.3.8) Problem Statement

Millions are spent on herbicides and insecticides by South African wheat farmers to produce tons of cereal per year. It is believed that aphid feeding on cereal plants can lead to widespread damage and extensive loss of yield. The development of resistant cultivars to reduce yield losses, however, may be challenged by the outbreak of new Russian Wheat Aphid biotypes. Thus, the development of new resistant cereal strains is of paramount importance to the national and international cereal industry.

1.3.9) Hypothesis

RWA-SA2 induces specific responses in Tugela Dn which may cause depletion in the resistance of Tugela Dn against RWA-SA2.

1.3.10) Aim

To gain a greater understanding of the resistance mechanism activated by the RWA resistance gene *Dn*1 in response to RWA-SA2 infestation.

1.3.11) Specific objectives

- To evaluate whether RWA-SA2 damages/compromises the photosynthetic mechanisms of wheat by measuring chlorophyll fluorescence of infested RWA-SA2 susceptible and resistant wheat cultivars.
- To identify exclusive expressed proteins during RWA-SA2 infestation on RWA-SA1 susceptible and resistant wheat cultivars.
- To identify possible signalling pathways induced in cereals by phloem feeding through profiling the proteome of wheat during RWA-SA2 infestation.

CHAPTER 2

Changes in Chlorophyll Fluorescence Induction Kinetics in Tugela and Tugela Dn Infested With Russian Wheat Aphid-SA2

2.1) Introduction

Plants require CO_2, H_2O and sunlight to provide their energy requirements which are synthesized in a chemical process called photosynthesis. This process occurs within plant cells in specialized structures known as chloroplasts. Two sets of reactions occur with this process- the light dependent reactions and the Calvin cycle (Baker, 2004). Chloroplasts house small disc-like structures called thylakoids, where the conversion of light energy to chemical energy is initiated. Thylakoids contain pairs of photosystems called photosystem I and photosystem II that work in tandem to produce the energy that will be used in the stroma to manufacture sugars. These photosystems consist of a network of accessory pigment molecules and chlorophyll, the molecules that absorb the photons of light. Within the pigment molecules, the absorbed light energy excites electrons to a higher state. Photosystems will channel the excitation energy gathered by the pigment molecules to a reaction centre chlorophyll molecule which will then pass the electrons to a series of proteins located on the thylakoid membrane (Flexas, 2009).

Chlorophyll absorbs light more effectively in the red and blue parts of the visible spectrum. Chlorophyll fluorescence is the result of light that is re-emitted at a longer wavelength after being absorbed by chlorophyll molecules at shorter wavelengths. By measuring the nature and intensity of variable chlorophyll fluorescence – which is only observed in chlorophyll a in photosystem II – Plant Physiologyogy can be investigated (Baker, 2004). Variable chlorophyll fluorescence allows one to investigate the light dependent reactions of plants, plant photo-protection mechanisms, dissipation of heat, photosynthetic reactions as well as measurements of most types of plant stress (Baker, 2004).

Much greater interest lies in chlorophyll fluorescence since photosystem I only emits chlorophyll fluorescence at a much lower level than photosystem II and it is also not variable (Schreiber, 2004). Variable chlorophyll fluorescence was first detailed by Kautsky in 1931 and became known thereafter as the Kautsky effect (Kautsky, 1931). Photosystem II reaction centres are comprised of two varieties, Quinone A (Q_A) and Quinone B (Q_B) which are involved in energy transfer and affect variable chlorophyll fluorescence (Zhu, 2005). However, it should first be mentioned that D_1 and D_2 pigment protein complexes coordinated the specialized chlorophyll a photoactive centre structure P680 in the photosystem II reaction centre (Papageorgiou, 2004).

Energy may either be transferred to Q_B reducing reaction centres capable of being used in photochemistry or to Q_B non-reducing reaction centres that are not capable of transferring their energy to other reaction centres, and therefore re-emit the energy as non-variable chlorophyll fluorescence at a low level (Zhu, 2005). An increase in the minimum fluorescence, F_0, is a result of a higher number of Q_B non-reducing reaction centres which ultimately results in a decrease of F_V/F_M (Zhu, 2005). F_V/F_M is a normalized ratio which allows one to compare plant samples and is the most used chlorophyll fluorescence measuring parameter.

$F_V/F_M = [F_M - F_0]/F_M$

F_M represents the maximum fluorescence from the Kautsky curve (when all PSII reaction centres are in an oxidized open state) after proper dark adaptation. Once exposed to light, all available PSII reaction centres close or become chemically reduced.

F_0 is the fluorescence emitted before any Q_A has been chemically reduced. According to Papageorgiou (1975), chlorophyll a fluorescence transients' measurements have served as useful markers of plant tolerance to unfavourable environmental conditions.

The objective of this section was to ascertain whether chlorophyll fluorescence induction kinetics measurements would shed light into the physiological mechanisms of RWA-SA2 damage and how the photosynthetic capacity of the plant becomes compromised over time.

2.2) Materials and Methods

2.2.1) Plant Material and Insects

Tugela and Tugela Dn seeds were planted according to the method of Burd *et al*. (1993) with modifications, directly into a mixture of potting soil, vermiculite and sandy soil (2:1:2) approximately 1cm deep. Each 10 x 10cm pot contained three seeds which were grown under set conditions in a controlled chamber at the Botany Department, University of Fort Hare. The conviron was maintained at a constant temperature of 22°C (± 1°C) at a relative humidity of 70% with a 16hr photoperiod. Plant nutrient status was maintained with a 1g/L solution of hygrofert mix which was administered every three days. Test plants were infested with 10 apterous *D. noxia* (SA2) at the two leaf stage and both infested and non-infested control plants were housed in separate insect cages within the conviron. Colonies of RWA-SA2 were maintained on Scheepers using Pwlio (Conviron S1OH; Controlled Environments Limited, Winnipeg, Manitoba Canada; Analytical Scientific Instruments CC South Africa) in aphid cages (Figure 2.1) until used to stress plants. The Scheepers plants were replaced weekly to ensure succulent hosts for the aphids. The chamber was set at 22ºC, 2 Lamps (0352µMOL), 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).

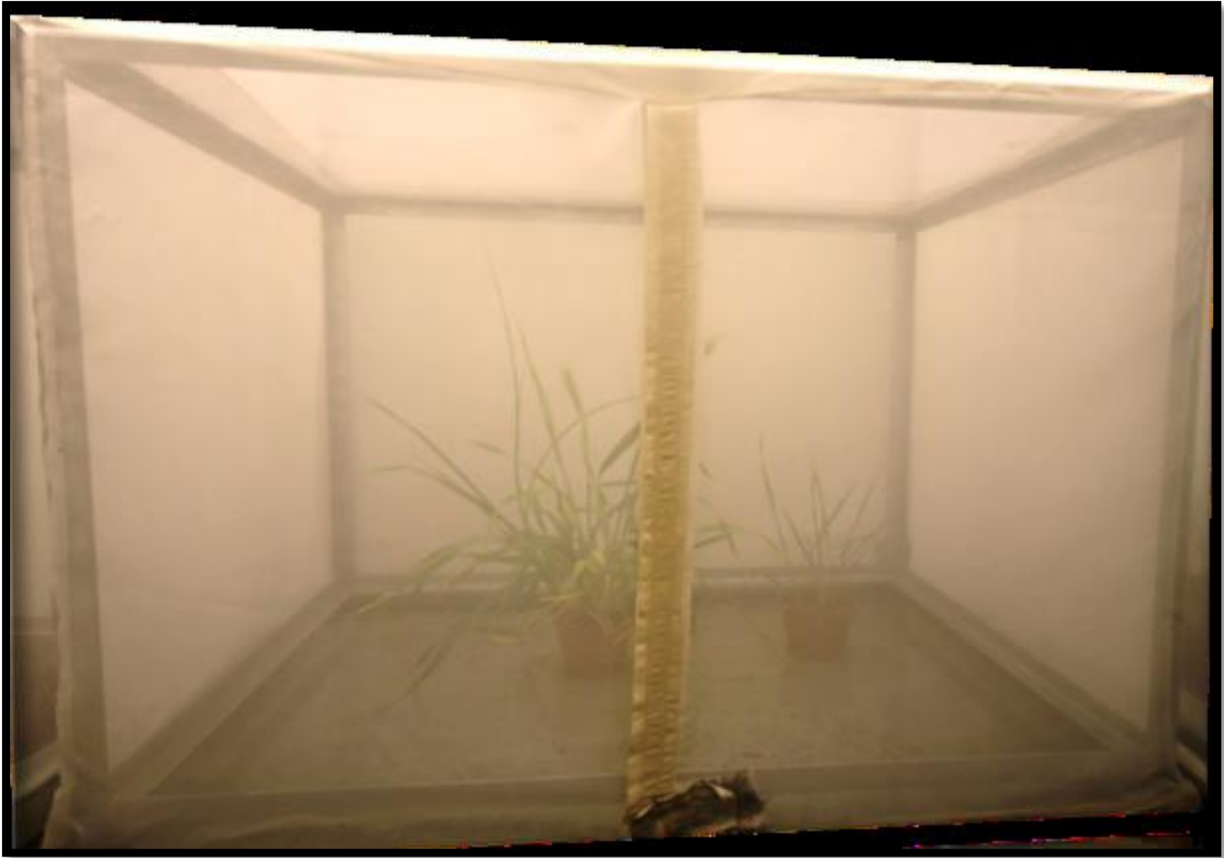


Figure 2.1: Aphid cage containing colonies of RWA-SA2 maintained on Scheepers wheat plants

2.2.2) Chlorophyll Fluorescent Kinetics

Chlorophyll "a" kinetics transients were measured using the LcPro integrated Fluorometer and photosynthesis system from OPTI-SCIENCES (ADC Bio-Scientific Ltd). Triplicate measurements were taken from the adaxial leaf surface of the main tiller for each of the test and control plants from day 0 to day 9. The primary fluorescence parameters, F_0 (non-variable fluorescence), F_m (maximum fluorescence), F_v (variable fluorescence), F_v/F_m (photochemical efficiency of photosystem II) as well as G_S (stomatal conductance) were measured at 21°C temperature, constant ambient CO_2.

2.3) Results and Discussion

2.3.1) Physiological responses of Tugela and Tugela Dn in response to RWA-SA2 feeding

Visible manifestations of RWA-SA2 feeding damage were assessed by examining chlorosis and leaf roll on each experimental plant for the entire time-course study (data not shown). The population growth rate for RWA-SA2 was generally exponential on both wheat cultivars, Tugela Dn and Tugela (susceptible) but lagged more on the resistant cultivar (Tugela Dn). Jimoh *et al.* (2011) reported that RWA-SA2 breeds faster on resistant and non-resistant barley lines. At day 9, Tugela susceptible plants were more profoundly infested by RWA-SA2 as compared to the resistant cultivar, Tugela Dn. Tugela Dn appeared healthy with only a few isolated chlorotic or necrotic spots at day 9 while the susceptible cultivar showed extensive chlorosis and necrosis with no possible recovery. This compliments the 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 more especially to the susceptible cultivar. Van der Westhuizen and Pretorius (1995) reported that Tugela Dn metabolically has a better ability to survive the stress condition imposed on it by *D. noxia* probing than Tugela due to a higher proline content and subsequent maintenance of chloroplast integrity and photosynthesis. Furthermore, with the resistant cultivar displaying 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). In addition, Nkongolo *et al.* (1990) reported that resistant strains are better able to cope with infestation and show less physical damage.

2.3.2) Changes in Chlorophyll a Fluorescence Induction Kinetics in Tugela Dn and Tugela infested with RWA-SA2

Results obtained on the primary chlorophyll fluorescence transients for Tugela and Tugela Dn hosts infested with RWA-SA2 indicate that although both wheat isolines (Tugela and Tugela Dn) exhibited aphid injury (Figure 2.2). Tugela as expected appeared to be more affected than Tugela Dn and at an earlier stage. It would appear that the chlorophyll concentration in the uninfested Tugela leaves were significantly higher than RWA-SA2 infested Tugela leaves from 120 hours onwards. The initial response of infested Tugela leaves against aphid attack (1 hour to 120 hours) is most probably due to a defense response by the plant. Tugela Dn infested leaves on the other hand appeared to have thrived quite well with negligible chlorophyll concentration loss. Chlorophyll decline indicates that aphid feeding adversely affected the plant. The chlorophyll concentrations in the aphid infested resistant isoline (Tugela Dn) were similar to levels observed in the respective un-infested Tugela Dn plants. This is confirmed with chlorophyll fluorescence transients' data with having little effect on chlorophyll fluorescence kinetics (Figure 2.2A) of the resistant entry. The susceptible entry (Figure 2.2B) showed that *D. noxia* caused a decrease in maximal (F_m) and variable (F_v) fluorescence resulting in a decreased photochemical efficiency of PSII (F_V/F_M). Kruger and Hewitt (1984) reported similar findings in which *D. noxia* infestation leads to a drastic reduction in chlorophyll content. This effectively results in reduced photosynthetic ability (Fouché *et al.*, 1984) and when combined with the characteristic leaf rolling that occurs, causes a considerable loss of effective leaf area of susceptible plants (Walters *et al.*, 1980) which was characteristic of the Tugela susceptible plants observed in this experiment. The results reported herein also appear to be in accordance with the results of Krause *et al.* (1982), Horton (1983) and that of Burd and Elliott (1996). However, they have worked with barley and different wheat germplasms and found that the reduction in the photochemical efficiency of PSII was caused primarily by a decrease in the variable fluorescence. Their findings also suggest that this reduction in photochemical efficiency is attributed due to the apparent over-reduction of Q_A. The reduced F_m (fluorescent intensity) was also thought to be a result of energy dependent fluorescence quenching related to photo-induced acidification of the thylakoid space or by structural changes in the PSII complex.

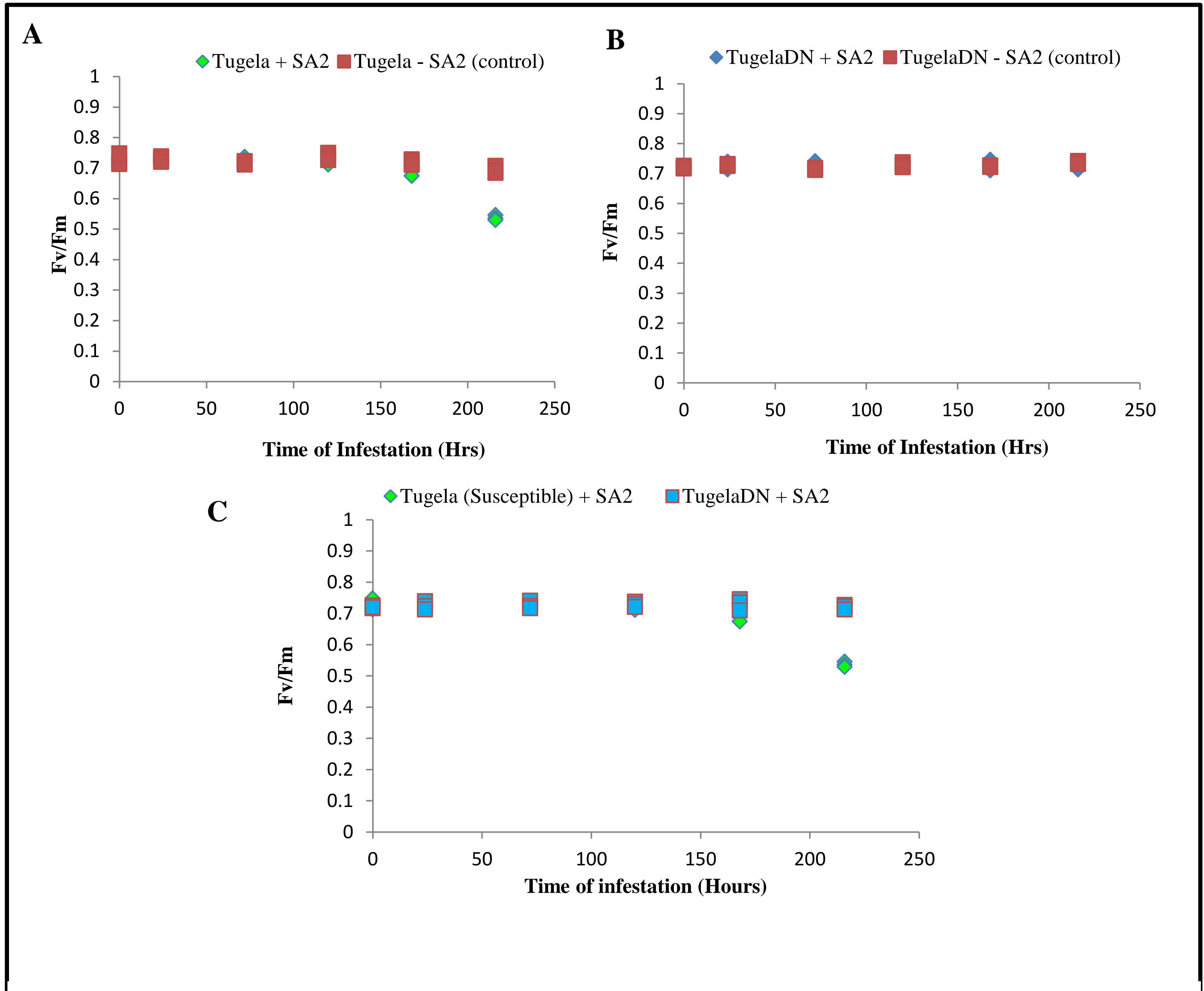
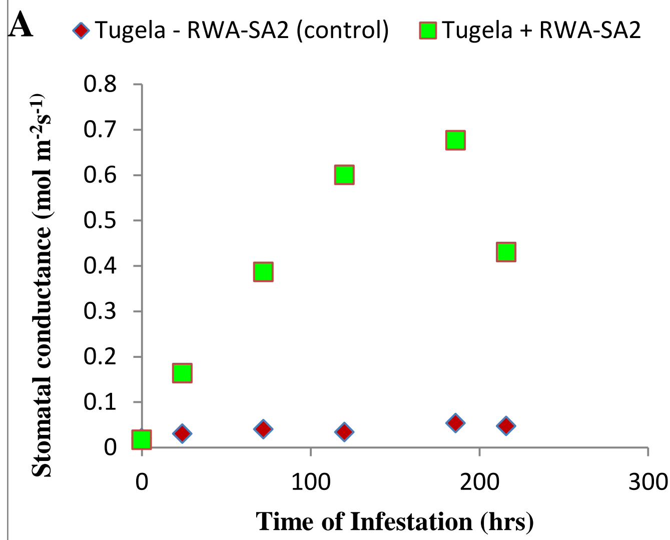
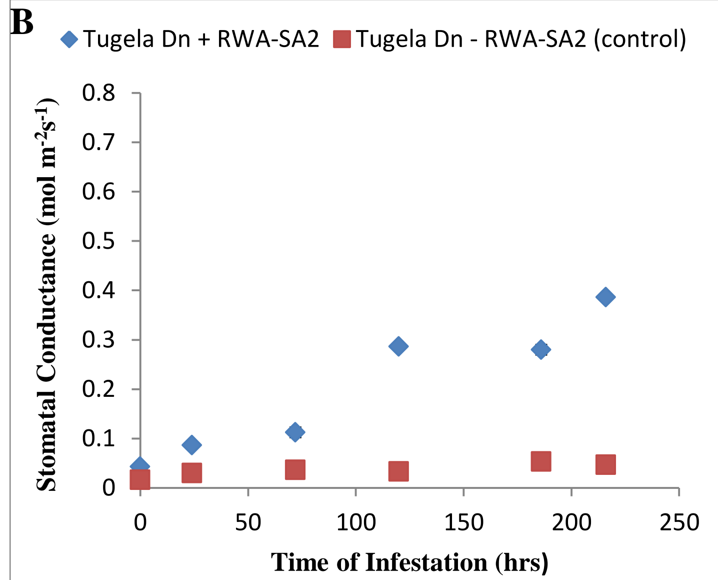


Figure 2.2: Progress curves of the photochemical efficiency of photosystem II. Triplicate readings (n=3) were taken at times: 0, day 1, day 3, day 5, day 7, and day 9. [A] - Tugela infested with RWA-SA2 against Tugela uninfested (control). [B] - Tugela Dn infested with RWA-SA2 against Tugela Dn uninfested (control). [C] - Tugela infested with RWA-SA2 against Tugela Dn infested with RWA-SA2.

2.3.3) Effect of RWA-SA2 feeding on Stomatal Conductance

In all the test plants, aphid feeding induced significant increase in the stomatal conductance in Tugela. Stomatal conductance (Figure 2.3) increased from 120% to 400% after 24hrs and 168hrs respectively, compared with that of aphid free plants. In Tugela Dn, stomatal conductance increased from 111% to 150% after 24hrs and 216hrs respectively, relative to their controls. It is commonly assumed that stomata have evolved for the purpose of controlling water loss from plants while allowing photosynthesis to proceed (Jones, 1997). Stomatal conductance was enhanced considerably in Tugela by RWA-SA2 feeding suggesting increased stomatal apertures. However, stomatal conductance dropped after 216hrs in Tugela which is a result from damage to the leaf tissues.





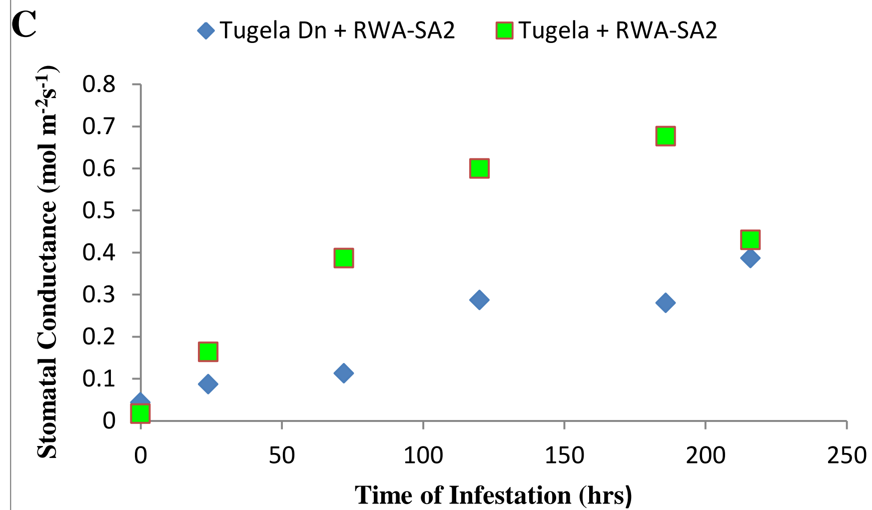


Figure 2.3: Progress curves of the Stomatal conductance. Triplicate readings (n=3) were taken at times: 0, day 1, day 3, day 5, day7, and day9. [A] - Tugela infested with RWA-SA2 against Tugela uninfested (control). [B] - Tugela Dn infested with RWA-SA2 against Tugela Dn uninfested (control). [C] - Tugela infested with RWA-SA2 against Tugela Dn infested with RWA-SA2.

A study conducted by Van Der Westhuizen and Pretorius (1995) concluded that changes in the chlorophyll, protein, free amino acid, proline levels and respiration rate in response to *D. noxia* infestation indicate that a stress condition is induced in both susceptible and resistant wheat plants by *D. noxia* feeding. The unique changes in resistant wheat, especially the marked increase in the total free proline content, seems to contribute to the plants improved ability to cope with *D. noxia* infestation and therefore survive. Proline is known to play a protective role for membrane systems under stress, thus, membranes in resistant plants remain intact and photosynthesis can proceed relatively normally as opposed to susceptible plants where the chloroplasts are damaged.

In concluding, chlorophyll fluorescent transient parameters that were measured appeared to be consistent with the associated plant resistance status. The results presented herein were consistent with the findings of Krause *et al.* (1982), Horton (1983) and Burd and Elliot (1996) in which we have reported a decrease in F_m and F_v resulting in a decreased photochemical efficiency of photosystem II (PSII) (F_V/F_M) for Tugela (susceptible), whereas total chlorophyll concentrations was not significantly affected in Tugela Dn (resistant cultivar). However, a study by Rafi *et al.* (1996) produced contradictory results, in which they reported that susceptible plants have similar chlorophyll concentration levels as their respective uninfested plants after exposure to *D. noxia,* whereas the resistant plants infested with *D. noxia* have reduced levels of chlorophyll when compared with uninfested plants. Heng-Moss *et al.* (2003) in a complimentary study compared chlorophyll and carotenoid concentrations among RWA-infested wheat lines found that their resistant isoline (Betta-Dn2) showed minimal chlorophyll loss even after 13 days of aphid feeding. This thesis provides further evidence to support this, despite using a more virulent RWA biotype (RWA-SA2). This suggests that Tugela Dn can compensate for prolonged aphid feeding damage. Furthermore, the results of this investigation also suggest that the damage imposed by RWA-SA2 goes beyond the simple removal of photosynthate from the plant. The substantial drop in F_V/F_M suggests that antennal chlorophyll complexes disengages from photosystem II reaction centres which could ultimately result in "cell-bleaching" (chlorophyll photo-oxidation) (Burd and Elliott, 1996). Physiologically, the quantum yield of PSII photochemistry is relative to the F_V/F_M ratio and highly correlates to the quantum yield of net photosynthesis (Adams *et al.*, 1990). Observation on the virulence effect indicates that RWA-SA2 inflicted severe and extensive chlorosis and leaf-roll on the susceptible cultivar. No visible damage during the annotated time-frame was observed for the resistant entry

which further provides evidence that the resistant entry can withstand aphid feeding damage for longer periods. Further investigation was needed (Chapter Four) to determine if changes in chlorophyll fluorescence transients would be a reliable method for evaluating plant resistance to *D. noxia*, particularly with respect to different mechanisms of host plant resistance.

CHAPTER 3

Optimization of 2-D Gel Electrophoresis Method

3.1) Introduction

Two dimensional gel electrophoresis (2DE) is a powerful proteomics tool that is used for the purification and characterization of proteins (Chinnasamy and Rampitch, 2006). It can be used to visualize and map the proteome of a given tissue or organ sample. The use of such a technique facilitates the resolution of complex protein mixtures, which allows for identification of differential protein regulation during various conditions within the life of a given tissue, organ or plant (Finnie *et al.*, 2002). The power of such a technique employs the ability to separate complex protein mixtures based on their net charge and molecular mass. First dimension separation occurs on the basis of net charge in which the purified protein mixture of interest is focused on an immobilised pH gradient, causing proteins to migrate towards their respective isoelectric points under an applied voltage. Usually a high voltage ramp is necessary for proteins to migrate along the IPG strip and resolve at their individual isoelectric points (Gòrg *et al.*, 2000). The isoelectric point of a protein is a point where the protein in question possesses an overall net zero charge. Separation in the second dimension relies on a difference in mass between the different proteins by using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Herbet *et al.*, 1997; Gòrg *et al.*, 2000). The different proteins resolve as spots on the second dimension gel and can subsequently be identified and analysed by using either mass spectrometry (MS) or N-terminal sequencing coupled with bioinformatics tools and software such as PDQuest™ or Progenesis PG 200 V 2006 (Skylas *et al.*, 2005).

3.2) Materials and Methods

Wheat seeds (*Triticum aestivum* L) cv, Tugela and Tugela Dn were obtained from the Agricultural Research Council, Small Grain Institute (Bethlehem, South Africa). *Dn* genes confer resistance to the Russian Wheat Aphid (*Diuraphis noxia*, Mordvilko) and wheat cultivars bred to contain any one of these *Dn* genes or a number of them are said to be RWA resistant wheat cultivars (Liu *et al.*, 2005; Heyns *et al.*, 2006). Potting soil, vermiculite and hygrofert were purchased from Hygrotech, East London, South Africa and Mandoval vermiculite (Alrode) respectively. Total protein extraction kits, 2-D starter kits, 2-D clean-up kits, IPG Strips, 11cm criterion gels, Sypro Ruby gel stains as well as protein assay kits were purchased from Bio-Rad (USA). Bovine Serum Albumin (BSA) fraction V was purchased from Roche Diagnostics, (Germany) and liquid nitrogen was obtained from the Department of Chemistry, Rhodes University, (Grahamstown, South Africa).

3.2.1) Russian Wheat Aphid colony maintenance

Colonies of RWA-SA2 were maintained on Scheepers using Pwlio (Conviron S1OH; Controlled Environments Limited, Winnipeg, Manitoba Canada; Analytical Scientific Instruments CC South Africa) in aphid cages (see Figure 2.1 until used to stress plants. The Scheepers plants were replaced weekly to ensure succulent hosts for the aphids. The chamber was set at 22ºC, 2 Lamps (0352µmol), 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).

Overview of the procedure employed to optimise the 2DE protocol for the determination of differential protein expression in wheat leaf tissue

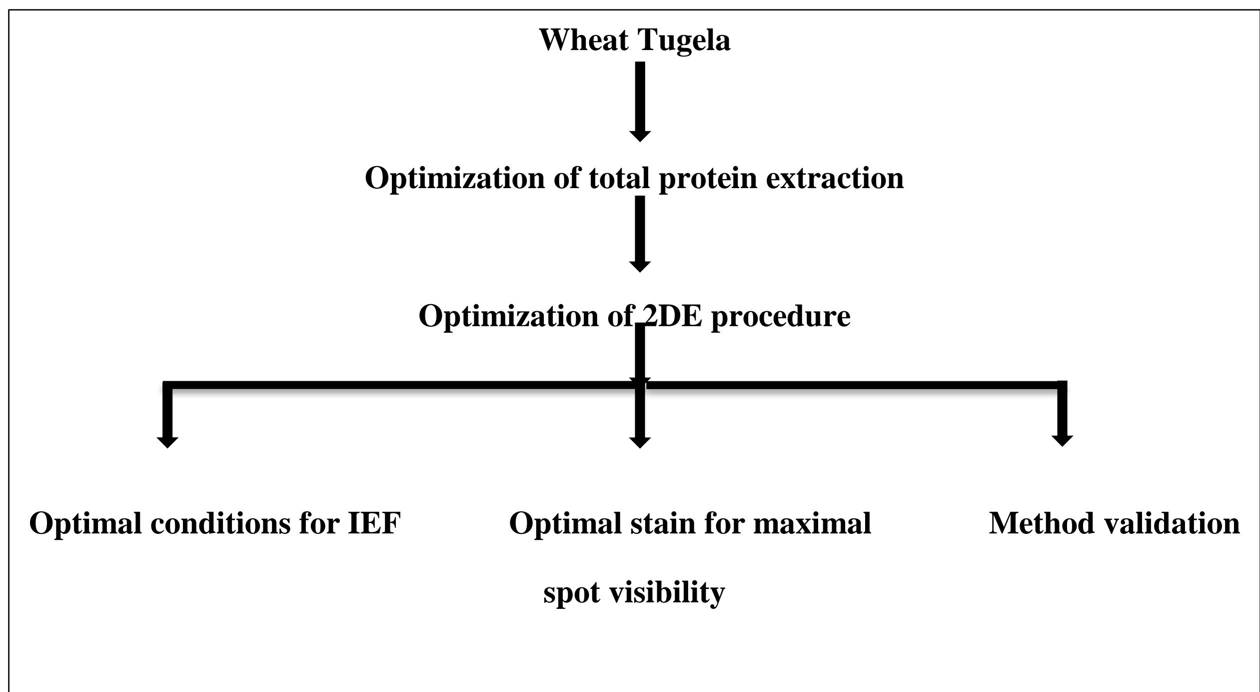


Figure 3.1: Overview of factors investigated during the optimization of the 2DE method for the determination of differentially regulated proteins in wheat leaf tissue in response to aphid feeding.

3.2.2) Cultivation of wheat Tugela and Tugela Dn plants

Wheat seeds were planted directly into a mixture of potting soil, vermiculite and sandy soil (2:1:2) approximately 1cm deep. Each 10 x 10cm pot contained three seeds which were grown under set conditions in a controlled chamber at the Botany Department, University of Fort Hare. The conviron was maintained at a constant temperature of 22°C (± 1°C) at a relative humidity of 60% with a 16hr/8hr day/night photoperiod. Plant nutrient status was maintained with a 1g/L solution of hygrofert mix which was administered every three days.

3.2.3) Total Protein Extraction

Leaves were harvested from plants when they reached the two leaf stage (14 days post germination). The leaves from each pot were pooled into well labelled 15mL 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 -80ºC freezer until further processing. Leaves were homogenized with a mortar and pestle under liquid nitrogen prior to extraction of total protein using the ReadyPrep^{TM} Protein Extraction Kit (Total Protein) (Bio-Rad, USA) as per kit manufacturer's instructions. Three extraction volumes were evaluated to determine the optimal leaf weight to extraction volume ratio. Briefly, 0.1g homogenized leaf tissue was suspended in either 200µl, 300µl or 400µl ReadyPrep^{TM} 2-D rehydration sample buffer 1 (1ml; 7M Urea, 2M thiourea, 1% w/v amidosulfobetaine (ASB-14) detergent, CHAPS, DTT, ampholyte, 40mM Tris base and 0.001% bromophenol blue) to which 10µl TBP reducing agent (200mM tributylphosphine in 1-methyl-2-pyrrolidine) was added. Samples were then sonicated in four bursts of 30 seconds each prior to centrifugation at 14000×g for 30min. After centrifugation, the pellet was retained, reduced and alkylated using the ReadyPrep^{TM} 2-D Starter kit (Bio-Rad, USA) as per the manufacturer's instruction, while the supernatant was discarded. The 2-D Starter kit is a great tool to run 2-D electrophoresis. It is useful for the reproducible of total cellular protein extracts from a given sample. This is due to the presence of the zwitterionic detergent, ASB-14 which acts as a powerful solubilising agent that also permits the extraction of membranous proteins making it particularly useful for 2DE applications. It contains all the reagents and an *E.coli* sample. The first step is to prepare the sample according to the manufacturers guidelines. Proteins were reduced and alkylated to disrupt protein disulphide bonds and in order to prevent different oxidation states in sample proteins (Herbert *et al.*, 2001; Taylor *et al.*, 2000). The optimal amount of extraction buffer for the purpose of protein extraction was determined by assaying the protein obtained from each of the three different volumes obtained and determining which was optimal in terms of maximum amount of protein extracted as illustrated in Figure 3.4. A 10 fold serial dilution of the samples was made. The manufacturer recommended a ratio of 2-3ml of sample extraction buffer per gram of plant leaf tissue. However, due to the limitation of plant biomass, 0.1g of leaf mass was used for the protein extraction.

3.2.4) Determination of protein content of wheat samples

The protein content of all wheat samples, both before and after the reduction-alkylation step was determined by means of the RC/DC protein assay (Bio-Rad, USA) which is a modification of the Folin-lowry assay (Lowry *et al.*, 1951).

Sample protein content was determined by interpolation from the standard curve using Bovine Serum Albumin (BSA) Fraction V as the protein standard (Figure 3.2). The Microfuge Tube Assay Protocol (1.5mL) was used as described. Briefly, Bovine serum albumin (BSA) stock (2mg/mL) was prepared by dissolving 0.002g 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 1.5mL microcentrifuge tubes. 125µL of RC reagent I added to each tube and the tubes vortexed and incubated at room temperature for 1 minute. After the incubation, 125µL 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 A1 (127µL) prepared by adding 5µL of reagent S to 250µL of reagent A was pipetted to each microcentrifuge tube, vortexed and incubated at room temperature for 5 minutes. The tubes were again vortexed and 30µL of the re-suspended protein in A1 was pipetted into three wells (triplicates) of the microtiter plate and 237µL of DC Reagent B added to each well. The absorbance was read at 750nm after 15 minutes of incubation in the plate reader (Synergy Mix, Biotek). BSA standard curves were created using Microsoft Excel® and the protein concentration of samples interpolated.

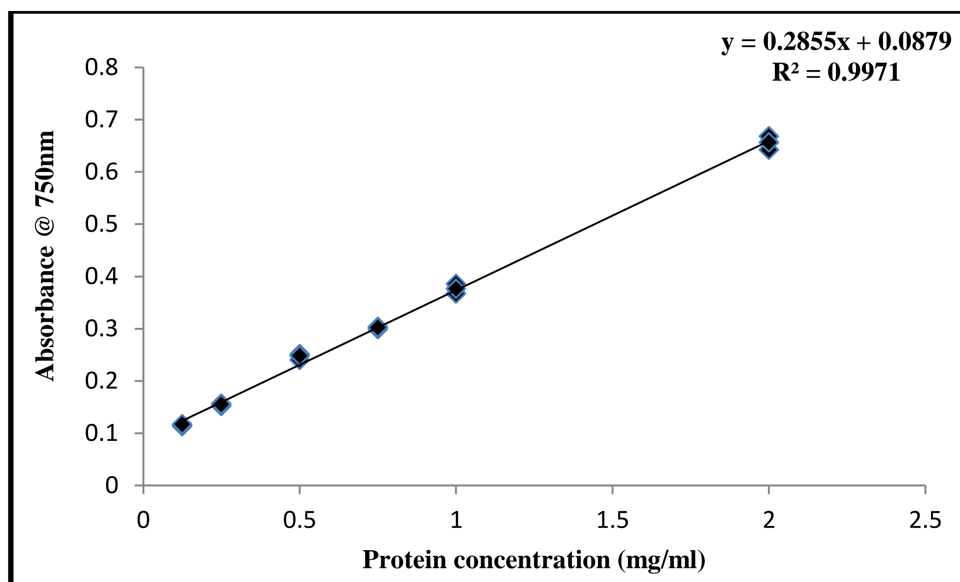


Figure 3.2: Sample standard curve generated using the RC/DC protein assay (Bio Rad) with Bovine Serum Albumin (Fraction V) stock solution of 2mg/ml. All readings were taken in triplicate and are shown on the figure.

3.2.5) Protein clean-up

Protein extracts were cleaned using ReadyPrepTM 2-D Clean up Kit (Bio-Rad, 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 (1-500µg) in a final volume of 100µL of 2-D starter Kit Rehydration/Sample buffer were transferred to 1.5mL microcentrifuge tubes. 300µL 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 300µL of precipitating reagent II. The tubes were mixed thoroughly by vortexing and centrifuged at maximum speed (21500 xg) for 5 minutes to form tight pellets. Tubes were removed promptly to avoid dispersing of pellets and the supernatants discarded by means of a 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. 40µL of wash reagent 1 was added onto the pellet and the tubes positioned in the centrifuge as before, centrifuged at maximum speed (21500xg) for 5 minutes and wash reagent discarded. Thereafter, 25µL of ReadyPrep^{TM} 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 (pre-chilled at -20°C for at least 1hr), and 5µL of wash 2 additive. The tubes were vortexed for 1 minute and incubated at -20°C for 30 minutes during which the tubes were vortexed for 30 seconds every 10 minutes. 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 5 minutes; when sufficiently dried it looked translucent. Each pellet was re-suspended by adding 250µL of 2-DE sample/rehydration buffer [8M urea, 2% CHAPS, 50mM 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 RC DC Protein Assay Kit (Bio-Rad, USA) as described above (Section 3.2.3. The supernatant was then used immediately to rehydrate IPG strips or stored at -80°C for later analysis.

3.2.6) Rehydration of IPG strips

IPG strips were rehydrated in Zoom® IPGRunnerO Cassettes prior to performing IEF. Broad-range pH 3-10 NL (11cm) ReadyStrip™ IPG strips were rehydrated overnight with 200µg of protein sample in a final volume of 185µL 2-D rehydration /sample buffer following the manufacturer‘s instructions; 185µL 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 peeled off and strips placed gently gel side down onto protein samples avoiding air bubbles being trapped. The strips were overlaid with 2mL of mineral oil to prevent evaporation during the rehydration process. The tray was covered and left to rehydrate on a level bench top overnight (11-16hr). Samples were run in triplicates to eliminate pot to pot variations that may occur.

3.2.7) 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 forceps at both ends of the channels covering the wire electrodes. Channels with same numbers as those used during rehydration were used. 8µL of nanopure water was pipetted onto each wick to wet it. With forceps, 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 2mL 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 3.1 using default temperature of 20°C with a maximum current of 50µA/strip.

Table 3.1: IEF Program for 11cm (pH 3-10)

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

On completion of the electrophoresis run, strips were removed from the focusing tray and transferred 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 parafilm and stored at -80°C.

3.2.8) Sodium dodecyl sulphate 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 6M urea, 2% SDS, 0.375M Tris-HCl (pH 8.8, 20% glycerol, and 2% (w/v) DTT] and II [20 mL of 6M urea, 2% SDS, 0.375M Tris-HCl (pH 8.8, 20% glycerol]. 4µL 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. 4mL 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 100mL 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™ TGX™, 4-20%, Bio-Rad, 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 3mm 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 200V for 35 minutes. The migration of the bromophenol blue present in the overlay agarose solution was used to monitor the progress of the run.

3.2.9) Determination of optimal staining method to maximise protein spot visibility

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 50mL SYPRO Ruby protein gel stain and wrapped in a container covered with aluminium foil. Gels were stained continuously with gentle agitation on a shaker overnight (16-18hrs). 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). The choice of a suitable stain is crucial for the success of 2DE experiments. Ideally a 2DE stain must have a broad linear range to allow for quantitative and qualitative analysis while also being sensitive enough to allow for visualisation of low abundance proteins. In addition to this, a successful stain should possess a wide linearity and dynamic range while being affordable to the researcher (Westermeier and Naven, 2002).

3.3) Results and Discussion

A 2DE protocol was developed to identify differential protein expression in Tugela and Tugela Dn wheat cultivars. This methods employed in the pilot study was considered sufficient to continue with the study on the differential protein expression during RWA-SA2 infestation on the two wheat isolines, Tugela and Tugela Dn. However, further optimisation was required to improve the reproducibility of gels. Commercially available kits were utilized to eliminate most of the variability due to human error during sample preparation for the 2DE runs.

3.3.1) Optimisation of protein extraction

In terms of manageability and the highest amount of protein extracted from 0.1g leaf sample, the 200µl of extraction buffer yielded similar amounts of total protein as the 300µl volume and slightly higher than the 400µl volume and was therefore used in all subsequent experiments (Figure 3.3).

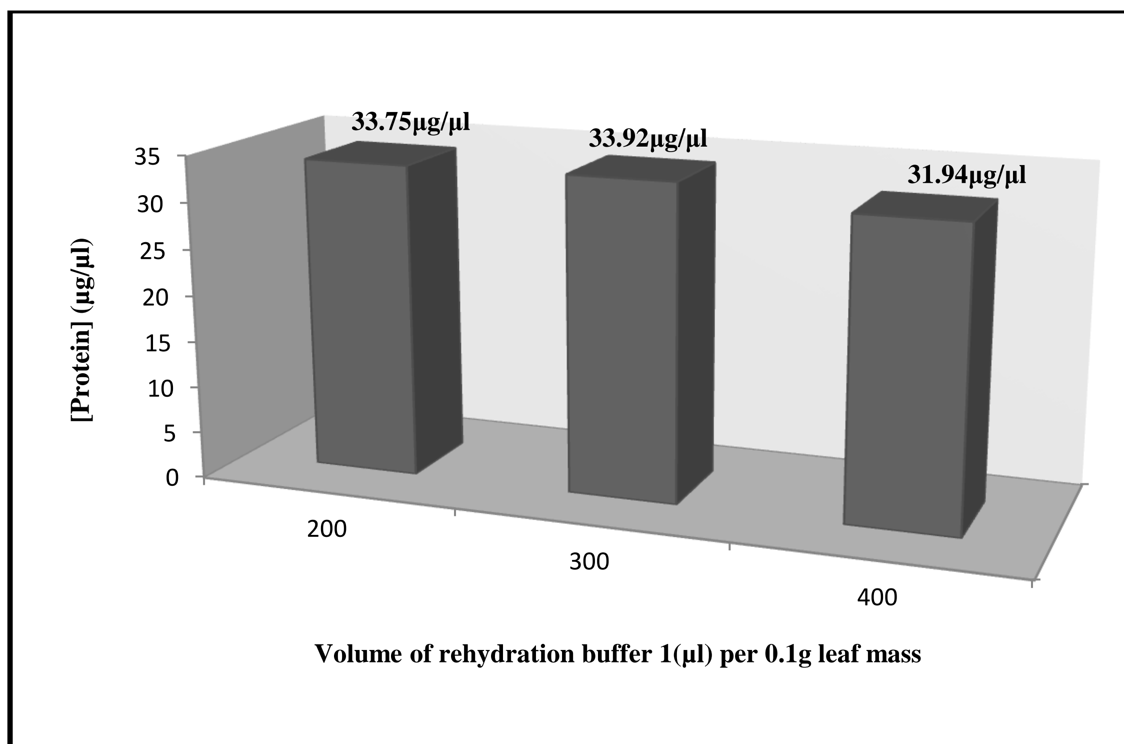


Figure 3.3: Protein yield from variable protein extraction buffer volumes (200µL, 300µL and 400µL) used, using the ReadyPrep^{TM} Protein Extraction Kit (Total Protein) from Bio-Rad (USA).

A protein load of 200µg was consistently applied per IPG strip and focusing performed as previously outlined. After focusing, IPG strips were applied to ReadyPrep™ 12% criterion gels for second dimension separation. Gels were stained with SyproRuby prior to analysis on the software. The optimal protein load for each system needs to be optimised according to the protein sample in question as well as the type of stain to be used in the subsequent step (Bjellqvist *et al.*, 1993). Care must be taken in loading protein samples onto rehydration trays as manufacturers guidelines stipulate that protein sample should be loaded in an even beat across the rehydration tray. The 2D clean-up kit indeed served as a handy tool kit. However, 2DE clean-up resulted in minute protein loss which was difficult to quantify due to the interference of the re-suspension buffer with the protein assay in addition to the difficulty of assaying the pellet after clean-up. The IPG strips were allowed to absorb most of the sample for an hour before 2ml to 3ml of mineral oil were added on top of each of the IPG strips to prevent them from drying out. Once the IPG strips were adequately rehydrated (11 to 16 hours), they were then focused by performing isoelectric focusing (IEF).

3.3.2) Optimisation of Staining

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. Furthermore, a successful stain should possess a wide linearity and dynamic range while being affordable to the researcher (Westermeier and Naven, 2002). Although Coomassie stain is cheaper and widely used protein stain, it was not sensitive enough for use in this study, as a protein stain for 2DE applications, as shown in Figure 3.4 (A). Figure 3.4 (A) further provides evidentiary support as only the most abundant proteins were visible on the second dimension gel stained with Coomassie. Greater care had to also been taken in order to destain protein spots corresponding to abundant proteins when destaining the gel background. The SyproRuby stain yielded markedly good spot visualisation and was found to be both cost effective and highly reproducible thus limiting the variability between gels and lowering the requirement for replicates during the course of the analysis. Figure 3.4 (B) shows a 2DE gel stained with SyproRuby stain and as one can see, spot visualisation of both abundant and less abundant proteins are quite prominent.

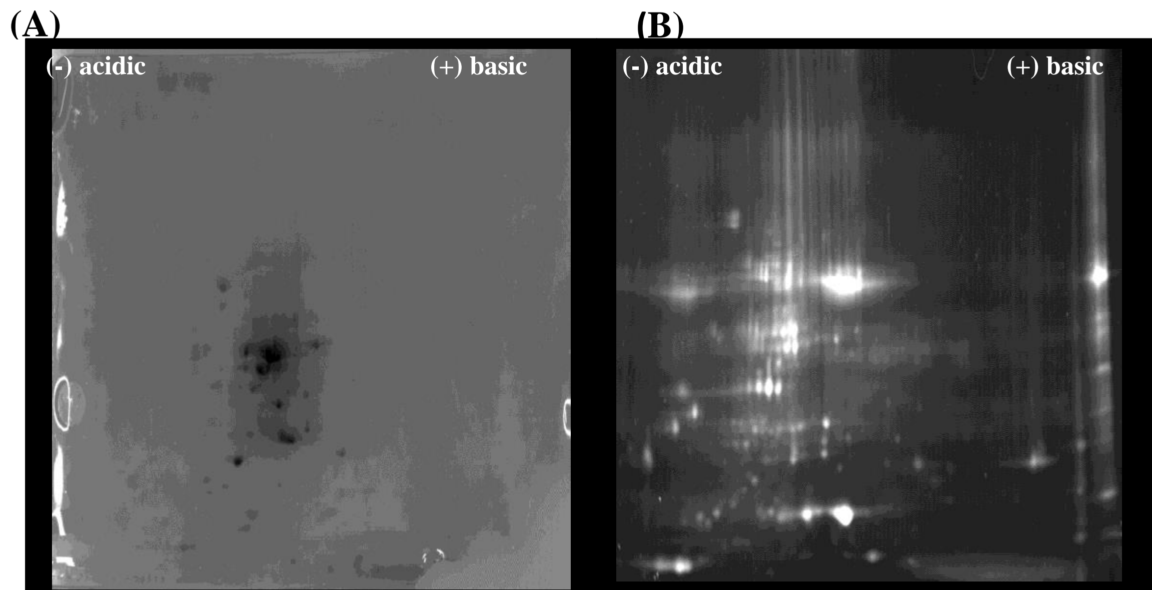


Figure 3.4: Total protein profile of wheat leaf in determining a suitable staining method to allow for the visualisation of a maximum number of spots on 2DE gels.

Wheat leaf samples at the two-week growth stage were resolved under optimised conditions. The wheat leaf protein concentration was maintained at ~1.35 mg/ml prior to clean-up and a final amount of 200µg protein was loaded on each gel. The IEF was performed using a Bio Rad PowerPac^{TM} HV power supply with a continuous ramp as outlined in section 3.2.6. The second dimension was resolved on ReadyPrep^{TM} 12% criterion gels for 35min. Gels were stained with Coomassie brilliant blue [A] and SyproRuby gel stain [B] respectively.

Research on the use of various protein gel stains, show that different stains interact differently with various proteins and recommend sensitive stains, such as silver or fluorescent stains, for 2-DE (Corthals *et al.*, 2000). However, silver stain is not Mass Spectrometry compatible and also has a limited linear range, becoming over-saturated at low levels of protein concentrations. Two stains were used in this study, for the optimisation process; they included Coomassie Brilliant Blue (R250) and SYPRO Ruby fluorescent gel stain.

From the gel picture (Figure 3.4) fewer protein spots were detected with Coomassie, an indication that Coomassie Brilliant Blue appears to only stain more abundant protein spots, which has also been reported by Carrol *et al.* (2000).

On the contrary, more protein spots were observed on the gel stained with SYPRO Ruby gel stain verifying its greater sensitivity than Coomassie. Although Coomassie is a less expensive staining method, it lacks the sensitivity of fluorescent stains, which was also reported by Louw (2007) who detected only a few spots on gels stained with Coomassie Blue-R-250. Fluorescent dyes, with their high detection sensitivity are therefore particularly useful for protein analysis, thus making SYPRO Ruby currently the most commonly used fluorescent protein stain in 2-DE. It is an endpoint stain with little background staining (high signal-to-noise characteristics) and it is widely used in conjunction with protein digestion of excised protein spots and MS analysis (Lopez *et al.*, 2000). Coomasie Blue R-250 typically has a detection limit of approximately 40ng 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).

CHAPTER 4

Identification of Exclusive Expressed Proteins during Russian Wheat Aphid-SA2 Infestation of Tugela and Tugela Dn Cultivars

4.1) Introduction

As mentioned previously, *D. noxia* is endemic to central Asia, southern Russia and countries bordering the Mediterranean Sea, Iran and Afghanistan (Durr, 1983; Hewitt, *et al.*, 1984) but now occurs in nearly all the major small grain production regions of the world except north eastern China (Robinson, 1992) and Australia (Hughes and Maywald, 1990). In South Africa, Walters (1984) first reported that *D. noxia* was a serious pest of wheat in 1978. Puterka *et al.* (1992) reported that *D. noxia* varies in its reaction to resistant wheat lines from different parts of the world. Due to the destructive nature of *D. noxia*'s feeding behaviour on crops, several methods have been devised to control the damages. These include the use of chemicals such as pesticides, biological methods – introducing natural enemies, cultural controls and the development of wheat resistant cultivars which appeared to be the most sustainable commercial and environmentally safe method to control *D. noxia* damage (Liu *et al.*, 2001; Tolmay, 2008; Dogimont *et al.*, 2010). In South Africa, Du Toit (1987) identified resistance of RWA in wheat and the first wheat resistant cultivar, Tugela Dn, which contained the *Dn* resistance gene was released in 1992 (Van Niekerk, 2001). Initially it was reported that resistant cultivars reduce aphid population by inhibiting RWA growth and reproduction (Tolmay and Mare', 2000). By the fall of 2005, Tolmay *et al.* (2007) confirmed the presence of a resistance breaking biotype of RWA in South Africa which is now understood to be resistance-breaking and virulent on existing RWA-resistant wheat lines. A population study conducted by Walton and Botha (2008) indicated that RWA-SA2 not only bred faster but also caused more damage to wheat lines than did RWA-SA1. RWA-SA2 appeared unaffected by the *Dn*1 resistance gene and posed a serious threat to small grain production in South Africa. This was further confirmed in a similar study conducted by Jimoh *et al.* (2011) in which they investigated the comparative effects of the feeding damage caused by two Russian wheat aphid (RWA, *Diuraphis noxia Kurdjumov*) biotypes, RWA-SA1 and RWA-SA2 three RWA-resistant barley (*Hordeum vulgare L.*) lines from the USDA-ARS, and used a South African non-resistant cultivar as control. They studied the relationship between aphid breeding capacity and the structural damage inflicted by the aphids. Both biotypes grew rapidly on all four barley lines with the population size and density generally lower on the resistant lines.

They also reported that RWA-SA2 bred significantly faster than the RWA-SA1 biotype and that feeding and water uptake-related damage sustained by phloem and xylem tissues of the resistant lines suggested that RWA-SA2 was a more aggressive feeder and caused considerably more cell damage than RWA-SA1.

Liquid chromatography mass spec (LCMS) has become extremely popular over the last few years as the sensitivity and accuracy of the systems have increased (Ong and Mann, 2005; Wilm, 2009). However, it has its drawbacks and one of the major limiting factors in the LCMS-based quantification via electrospray is the suppression of ions (Tang *et al.*, 2004). For this reason, the intensity of peptides is highly dependent on the quality of peptides that are ionized ionisation efficiency and the property of peptides being eluted (Tang *et al.*, 2004; Schmidt *et al.*, 2003). Knowledge of plant proteins and constituents and a comparison of these components between the two wheat isolines, would lead to better understanding of how *D. noxia* (RWA-SA2) damages wheat and possibly overcomes resistance mechanisms. Herein this section, a detailed 2D gel electrophoresis and LCMS analysis is presented that compares the wheat proteomes (Tugela vs Tugela Dn) at 3hrs, 6hrs and Day 7 post RWA-SA2 infestation to identify proteins that are exclusively expressed (Figure 4.2). The resulting peptide data and analysis were used to search locally restricted EST wheat databases to arrive at protein identifications and functions.

4.2) Materials and Methods

4.2.1) Experimental Approach

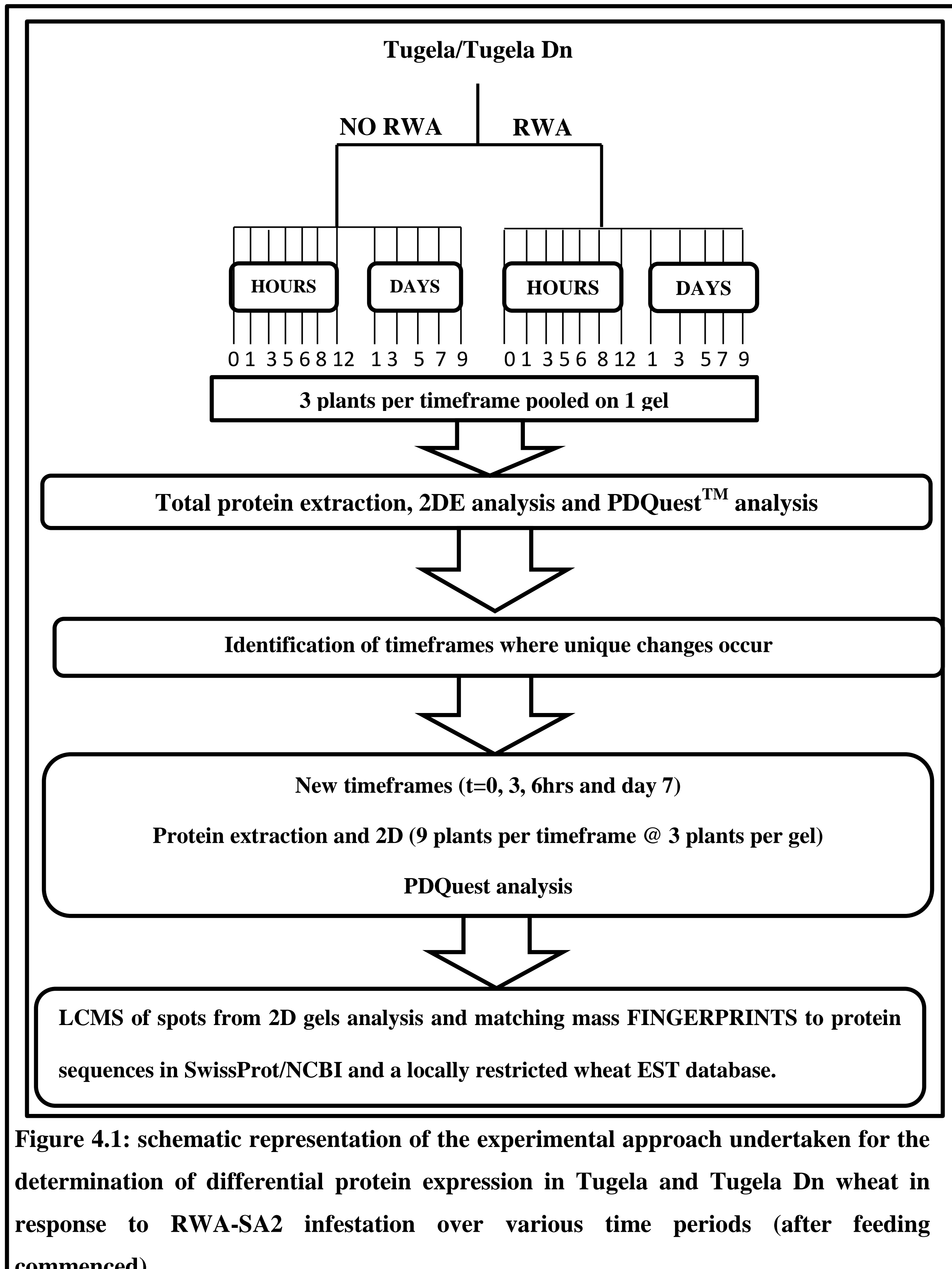


Figure 4.1: schematic representation of the experimental approach undertaken for the determination of differential protein expression in Tugela and Tugela Dn wheat in response to RWA-SA2 infestation over various time periods (after feeding commenced).

4.2.2) Cultivation of wheat Tugela and Tugela Dn plants

Wheat seeds were planted directly into a mixture of potting soil, vermiculite and sandy soil (2:1:2) approximately 1cm deep. Each 10 x 10cm pot contained three seeds which were grown under set conditions in a controlled chamber at the Botany Department, University of Fort Hare. The conviron was maintained at a constant temperature of 22°C (± 1°C) at a relative humidity of 60% with a 16hr/8hr day/night photoperiod. Plant nutrient status was maintained with a 1g/L solution of hygrofert mix which was administered every three days.

4.2.3) Total Protein Extraction

Total protein extraction was performed as outlined in Section 3.2.3.

4.2.4) Determination of protein content of wheat samples

The protein content of all wheat samples, both before and after the reduction-alkylation step was determined by means of the RC/DC protein assay (Bio-Rad, USA) as outlined in Section 3.2.4.

4.2.5) Protein clean-up

Protein extracts were cleaned using ReadyPrep^{TM} 2-D Clean-up Kit (Bio-Rad, USA) as outlined in Section 3.2.5.

4.2.6) Rehydration of IPG strips

IPG strips were rehydrated in Zoom® IPGRunnerO Cassettes prior to performing IEF. Broad-range pH 3-10 NL (11cm) ReadyStrip^{TM} IPG strips were rehydrated overnight with 200µg of protein sample in a final volume of 185µL 2-D rehydration /sample buffer following the manufacturer‘s instructions as outlined in Section 3.2.6.

4.2.7) Isoelectric focusing (IEF)

Isoelectric focusing was performed as outlined in Section 3.2.7.

4.2.8) Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Prior to separation of proteins by SDS-PAGE, the IPG strips were equilibrated in two buffers. 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 200V for 35 minutes as outlined in Section 3.2.8.

4.2.9) Gel Staining and Destaining

Gels were stained with SYPRO Ruby protein gel stain as outlined in section 3.2.9 and wrapped in aluminium foil. Gels were stained continuously with gentle agitation on a shaker overnight (16-18hrs). Gels to be analysed were analysed at CSIR, Pretoria (South Africa). Consumables used were: trypsin, Ammonium bicarbonate (NH4HCO3), Dithiothreitol (DTT), Iodoacetamide (IAA), Acetonitrile (CAN), Formic acid (FA), MS-H2O (Chromasolv), Protein Low-Bind tubes and Protein Low-Bind Deep well plates (DWP). See the section on general appendices for consumables preparation.

4.2.10) Excision of protein spots from polyacrylamide gels

Previously stained 2D gels with Sypro-Ruby were re-stained with colloidal Coomassie stain for approximately 3hrs with gentle agitation. Despite Coomassie being a less sensitive stain, the previously Sypro-Ruby stain enhanced detection of protein spots. This was necessary in order to view the protein spots of interest under a white box. After 3hrs of staining, the gels were destained with several volumes of Mili-Q water with gentle agitation. Gel spots of interest were then excised manually with a sterile blade diced into smaller gel pieces and placed into 0.5ml Protein Low-Bind tubes.

4.2.11) Washing and destaining of gel pieces

The method of Shevchenko *et al.* (2007) was adopted with minor modifications as follows: Approximately 200µl of 50mM (Ammonium bicarbonate) NH_4HCO_3/50% Methanol (MeOH) were added to the gel pieces and vortexed in a thermomixer for 20 minutes. The supernatant was discarded and the step was repeated. 100µl of 75% acetonitrile (ACN) was added to the gel pieces, vortexed in a thermomixer at 800rpm for 20 minutes and the supernatant discarded. The gel pieces were vacuum-dried to complete dryness for 20 minutes. At this stage, the gel pieces were translucent in appearance.

4.2.12) Reduction and alkylation of peptides in gel pieces

The reduction and alkylation step was necessary for low-level proteins (<1pmol). 25µl of 10mM DTT in 25mM NH_4HCO_3 was added to the translucent dried gel pieces, briefly vortexed and centrifuged at 800rpm in a Hermle Z100M centrifuge. The reaction was then allowed to proceed at 60°C in a water bath for 1 hour. Once the reaction was halted, the tubes were chilled to room temperature and 500µl of ACN was added. The reaction was incubated for a further 10min and the supernatant discarded after which 25µl of 55mM IAA in 25mM NH_4HCO_3 was added to the gel pieces. They were then vortexed and centrifuged at 800rpm and the reaction allowed to proceed in the dark at room temperature for 20 minutes. The supernatant was removed, discarded and the gel pieces were washed with 100µl of 25mM NH_4HCO_3 in Milli-Q water. This was once again vortexed for 10 minutes, centrifuged and the supernatant removed. The gel pieces were then dehydrated with 100µl of 25mM NH_4HCO_3 in 50% ACN. The step was repeated a second time and the gel pieces were vacuum-dried for 20 minutes. The samples were then ready to proceed with trypsin digestion.

4.2.13) Trypsin digestion of peptides in gel pieces

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.

Trypsin's ability to produce smaller average lengths as well as charge retaining residues at the C-terminus of the digested peptides makes it the preferred digestion method for modern reverse phase liquid chromatography and tandem mass spectrometric analysis. The gel pieces were rehydrated on ice with freshly prepared trypsin solution (~5 - 50μl) to barely cover the gel pieces. They were then monitored after 30 minutes to see whether all trypsin solution was absorbed or more had to be added. Rehydration was allowed to continue for a further 60 minutes. Once rehydration was completed, the Eppendorf tubes containing the gel pieces were centrifuged to which an additional 25μl of 25mM NH_4HCO_3 was added to keep the gel pieces wet during enzyme cleavage. The reaction was then incubated at 37°C overnight in a thermomixer.

4.2.14) Extraction of peptides

After overnight in-gel trypsin digestion of the protein samples, the aqueous digest solutions were transferred into clean 0.5ml Protein Low-Bind tubes. The remaining gel pieces were further rehydrated with 30μl of 50% ACN / 5% Formic acid to ensure maximal peptide extraction. These were then vortexed for 30 minutes in a thermomixer, spun and sonicated in a water bath sonicator for 5 minutes. The aqueous extract was then combined with the initial solution and the steps repeated again. The total digest solution (combined aqueous extract) was then vacuum-dried to complete dryness. Digests at this stage were ready to be analysed on the LCMS system.

4.2.15) Identification of peptides using the LCMS system

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 TripleTOF 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 and then separated on a 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 TripleTOF mass spectrometer was operated in the 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 at100 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 *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 above ≥ 99.9% confidence were reported.

4.3) Results and Discussion

4.3.1) 2DE gel analysis using PDQuest^{TM} basic software version 8.0

All 2DE gels were studied in triplicate in order to determine the number of visible spots as well as the spot variance between gels. Gels were grouped and analysed using the spot detection parameter wizard with a test settings sensitivity of 10. The faint and large cluster spots were selected from both the control and test gels to enable spot detection. The Gaussian model parameter was used while allowance was made for horizontal and vertical streak removal as well as a speckle filter set at 50 applied. In each case the gel corresponding to the control reaction for the given experimental group served as the master gel for the purpose of the software analysis. This software relies on manual identification of faint spots, large spots and the largest spot cluster, allowing for user input in terms of parameters. A Gaussian statistical model was applied to detect and fit spots while a local regression model used by the software was selected for data normalisation. A spot that occurred in every gel in the analysis set, both stressed and unstressed, at roughly the same intensity and was neither identified as 2-fold up or down regulated by the software – was selected and putatively identified to comprise a validation for the software's analysis of up or down regulated proteins.

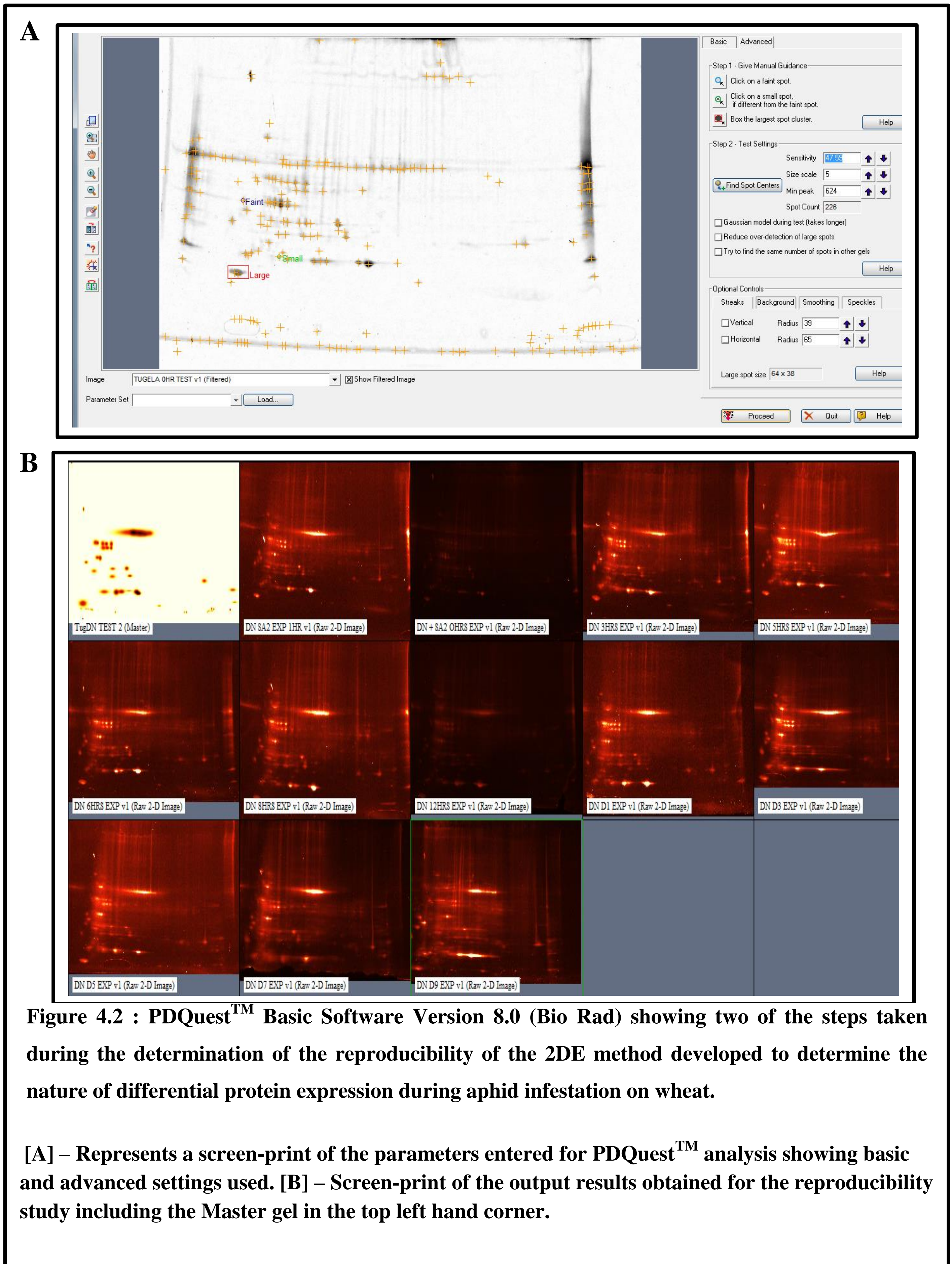


Figure 4.2 : PDQuest™ Basic Software Version 8.0 (Bio Rad) showing two of the steps taken during the determination of the reproducibility of the 2DE method developed to determine the nature of differential protein expression during aphid infestation on wheat.

[A] – Represents a screen-print of the parameters entered for PDQuest™ analysis showing basic and advanced settings used. [B] – Screen-print of the output results obtained for the reproducibility study including the Master gel in the top left hand corner.

4.3.2) Experimental Summary

Once the software completed its matching, an experimental output window was displayed containing an interactive chart (Figure 4.3), showing the number of matched protein spots on the gels within the analysis set based on the user settings. This report/output also allows one to change any necessary settings such as matching and normalization to possibly enhance the correlation coefficient ratio. This parameter (correlation coefficient) provides one with the level of variability within the analysis set. It was then decided to compare each test gel with the control per time frame within a given analysis set in the output results window to determine spot quantity. This was further enhanced by using the quantity graph report.

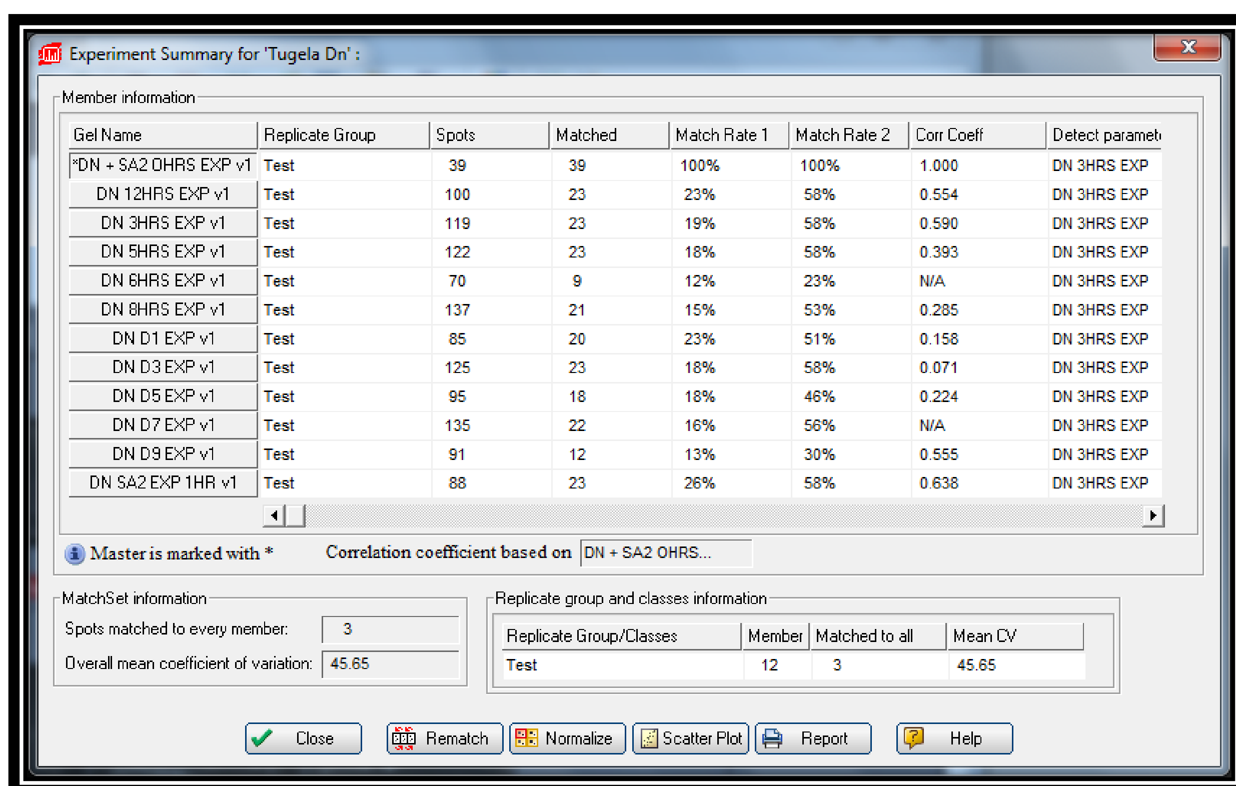


Figure 4.3: Experimental output window showing the number of matched protein spots on the gels

Input parameters for spot selection (faint and largest spots) in the PDQuest^{TM} software aided in the elimination of background hindrances that could resemble spots as well as the removal of streaks which resulted in the enhancement of actual protein spots detection. A match set was created that included all gel images within an experiment to allow for spot-to-spot comparisons across gels.

This match set served as the master gel. The master gel, created by the software is 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 4.2). Each gel in the experiment was then compared to the relevant master gel. The experiment summary table (Figure 4.3) 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 and finally, the correlation coefficient showed that individual gel images matched within the replicate.

The scatter plots (Figure 4.4) represents 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).

All treated gels were compared with their respective controls to see where proteins are exclusively expressed. The results obtained showed that unique changes occurred at 3hrs, 6hrs and Day 7 post infestation (Figure 4.7). The zero time frame (before infestation) served as a control to compare the peptide profiles of individual treatments. Peptide profiles of wheat leaf tissues from each treatment (a resistant and non-resistant line) were compared using the PDQuest^{TM} version 8.0 software and exclusively expressed proteins identified by MS and MS/MS searches of the NCBI/Swiss-Prot and a locally restricted wheat EST database.

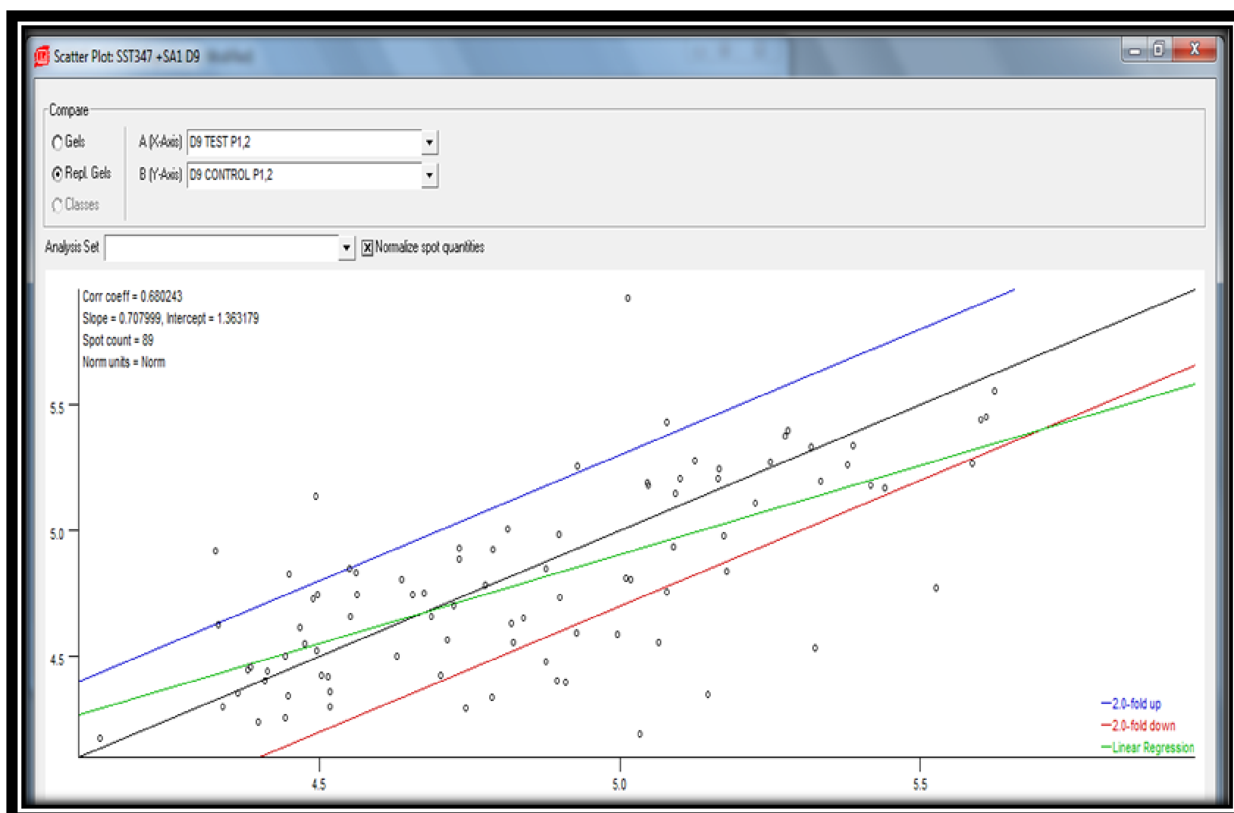


Figure 4.4: Linear regression of reproducibility of 2-DE gels in duplicate after PDQuest™ analysis. The blue and red lines shows the border for the two fold differences between protein spot quantities while the green line shows a linear regression that represents correlation between the two groups.

4.3.3) Quantity Graph Report

The quantity graph report gives a general trend to better understand how frequently or seldom have certain spots occurred, hence detecting unmatched spots or up/down regulated protein spots. Each bar in the histogram – which is an estimate of the probability distribution of a quantity variable – represents the spots quantity in a member of the matched set drawn in proportion to the highest bar. As soon as a spot was identified, it was catalogued as a unique SSP (Standard Spot) number which was displayed beneath each histogram and provides information on the location of the spot. Figure 4.4 is an example of a quantity graph report from one of the analysis set. Initially, single gels were run at all 12 time points in order to identify at which time points the most significant/unique spots were detected. These time points would then be used for running additional 6 replicates per timeframe (test and control) (see Appendix 1 for all quantity graph reports for the entire experiment).

Figure 4.5: screen-print of a quantity graph report obtained from one of the matched analysis sets.

Results obtained from the quantity graph reports were then used to construct a histogram, representing a summary of protein spots detected on gels of Tugela and Tugela Dn, with and without RWA-SA2 infestation (Figure 4.5). This data also provides one with a quantitative analysis of proteins involved in the defense mechanism of a particular wheat isoline. In order to have determined proteins exclusively expressed that are involved in a possible resistance mechanism, Tugela Dn (treated) was compared to its susceptible counterpart (Tugela treated).

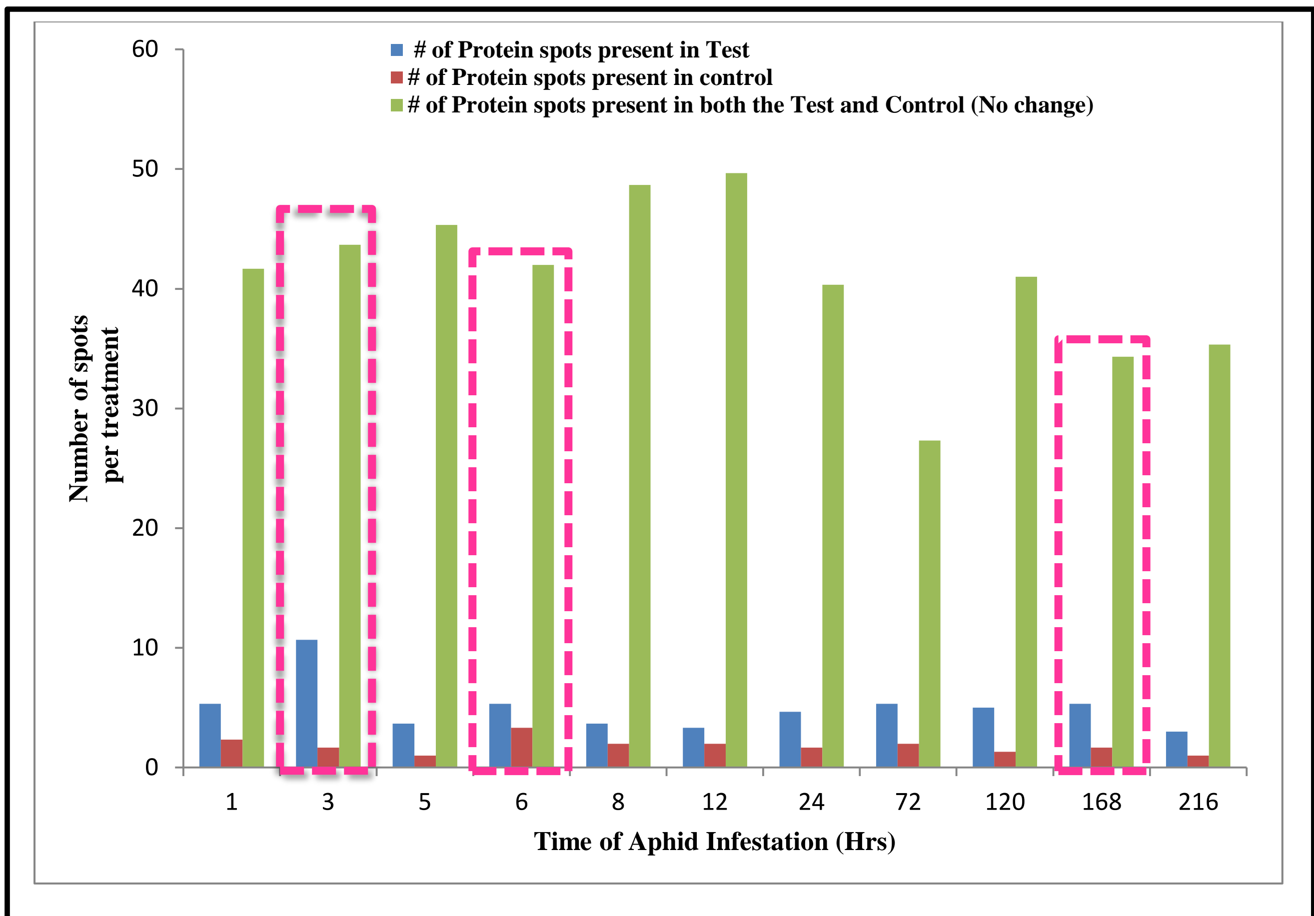
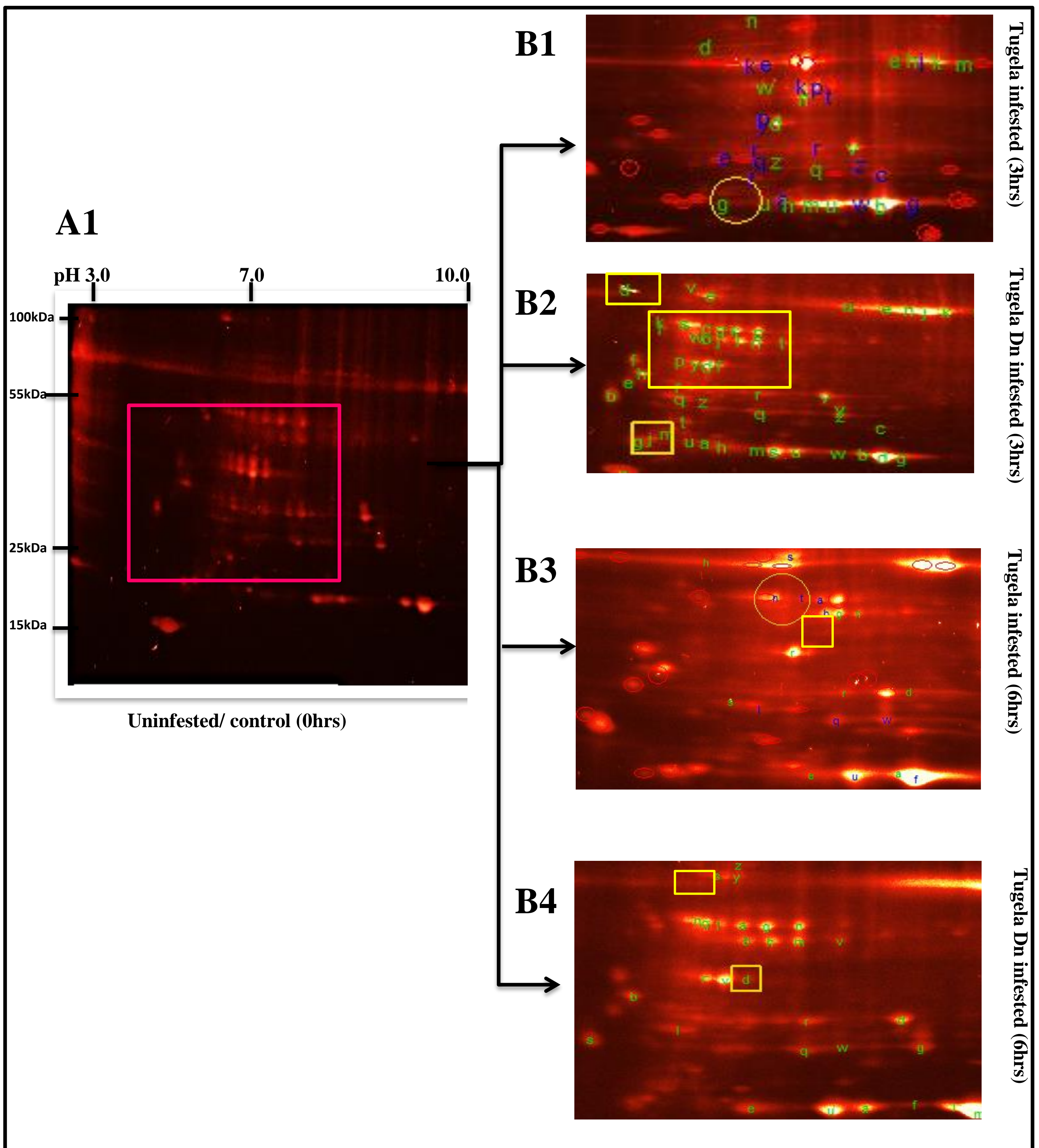


Figure 4.6: Summary of results obtained from the 2DE analysis of Tugela Dn with and without RWA-SA2.

Significant and unique changes were observed at 3hrs, 6hrs and day 7 (168hrs) post infestation and were selected for the additional replicates (Figure 4.6). Selection of these times was further supported by previous studies which reported that the first few hours of a plant's exposure to a given pest or pathogen is the most crucial in which the plant's defense responses such as the hypersensitive response (HR) and systemic acquired resistance (SAR) tend to come into play (Botha *et al.*, 2006; Moran *et al.*, 2002). The replicates gels from Tugela Dn infested samples were then compared to their respective Tugela (susceptible) infested samples at those given timeframes to possibly identify proteins involved in the resistance mechanism.

4.3.4) Identification of exclusively expressed protein spots to be excised

The aim of this study was to understand how wheat cultivars that are resistant to RWA-SA1, respond to RWA-SA2 infestation and whether RWA-SA2 overcomes the resistance mechanism of Tugela Dn. Therefore to identify the Tugela Dn proteins differentially regulated in response to RWA-SA2, 2D gels of protein extracts from 9 plants (3 plants per gel) of Tugela Dn infested with RWA-SA2 were compared to uninfested controls, over various time frames (3hrs, 6hrs and 7 days). These results were compared to differentially regulated proteins in infested and uninfested Tugela plants at 3hrs (Figure 4.7 [B2]), 6hrs (Figure 4.7 [B3 and B4] and 7days (Figure 4.7 [B5] and [B6]), to identify which of the differentially regulated proteins from Tugela Dn are related to the resistance mechanism of Tugela Dn.



A1
pH 3.0
7.0
10.0
100kDa
55kDa
25kDa
15kDa
Uninfested/ control (0hrs)
B1
Tugela infested (3hrs)
B2
Tugela Dn infested (3hrs)
B3
Tugela infested (6hrs)
B4
Tugela Dn infested (6hrs)

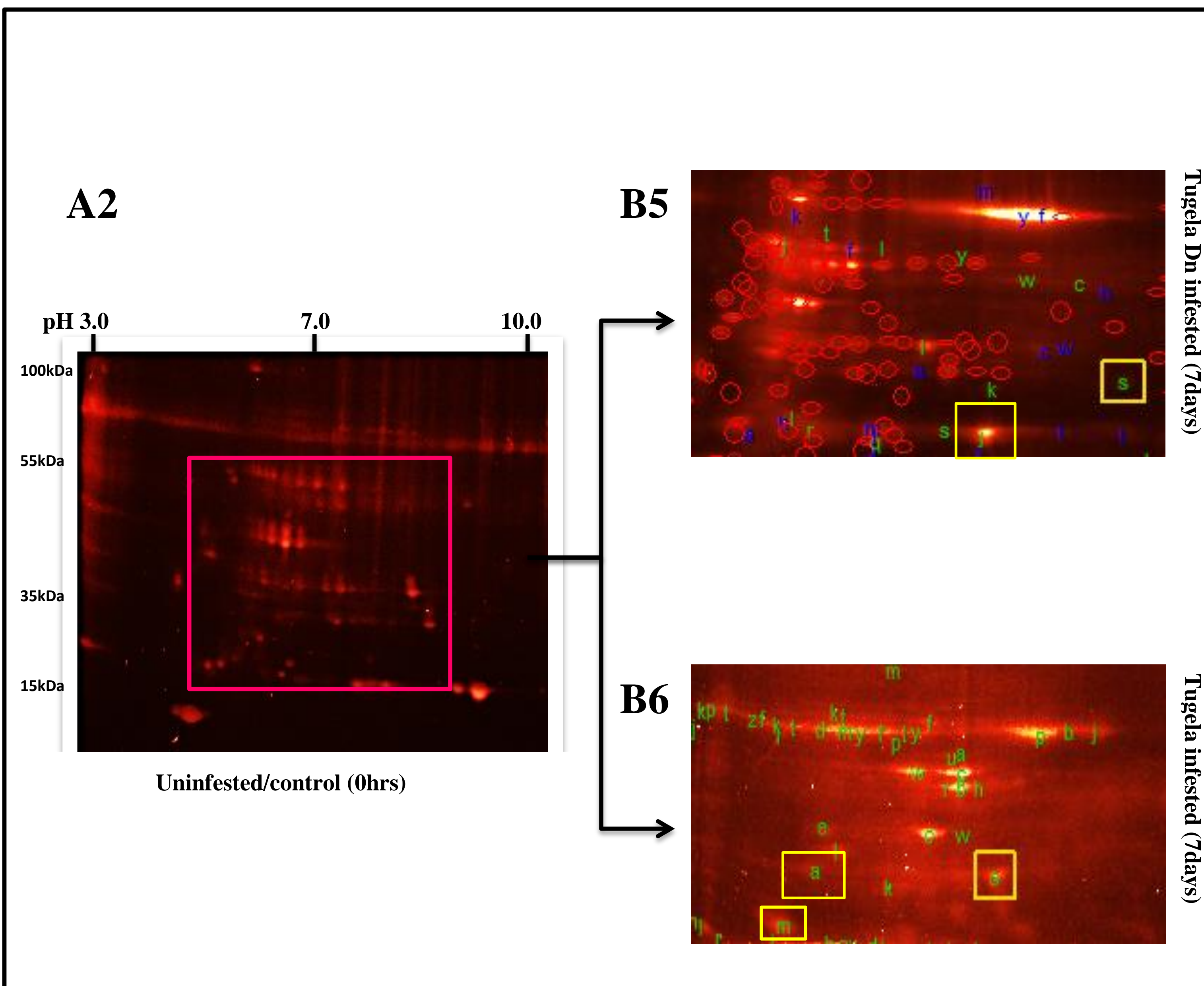


Figure 4.7: Summary of differential protein expression in both Tugela and Tugela Dn in response to RWA-SA2 phloem feeding.

Areas of most pronounced differential protein expression are boxed in pink [A1 and A2] in the figures on the left. Magnification of corresponding areas of RWA-SA2 stressed gels are inserted on the right [B1 – B6]. Detailed gel images and analysis are recorded in appendix 2 [A1 and A2] – wheat in the absence of aphid phloem feeding (control); [B1] – Tugela stressed with RWA-SA2 (3hrs post infestation); [B2] – Tugela Dn stressed with RWA-SA2 (3hrs post infestation); [B3] – Tugela stressed with RWA-SA2 (6hrs post infestation); [B4] – Tugela Dn stressed with RWA-SA2 (6hrs post infestation); [B5] – Tugela stressed with RWA-SA2 (Day 7 post infestation); [B6] – Tugela Dn stressed with RWA-SA2 (Day 7 post infestation). Exclusively expressed proteins of interest are boxed yellow. A detailed and thorough marking and description of these proteins will be discussed further on in this chapter.

4.3.4.1) Exclusively expressed proteins at 3hrs following RWA-SA2 infestation

12 spots were identified to be present in the control and in the Tugela Dn proteome at 3hrs after infestation but not in the infested Tugela proteome, indicating that these proteins were downregulated in the susceptible cultivar. These protein spots occurred in the pI range (pI 4 – 6) and molecular weight range 30 – 50kDa. These 12 proteins were identified by matching MS fingerprint to sequences in the SwissProt protein database, NCBI and the wheat EST database. These putative matches are detailed in Table 4.1. These proteins are largely involved in photosynthesis, metabolism, gene expression and regulation as well as structural support of the plant including: RuBisCO large subunit-binding protein subunit α, Ribulose bisphosphate carboxylase small chain clone 512, Fructose-1,6-bisphosphatase, Photosystem II D2 protein, Photosystem II protein D1, Dehydrin COR410, Phosphoribulokinase, ATP synthase subunit β, S-adenosylmethionine synthase, Phosphoglycerate kinase, Ribulose bisphosphate carboxylase large chain, Ribulose bisphosphate carboxylase small chain PW9, ATP synthase subunit α, Ribulose bisphosphate carboxylase small chain PWS4.3 and Sedoheptulose-1,7-bisphosphatase. Several proteins were identified to co-elute, albeit at a much lower abundance.

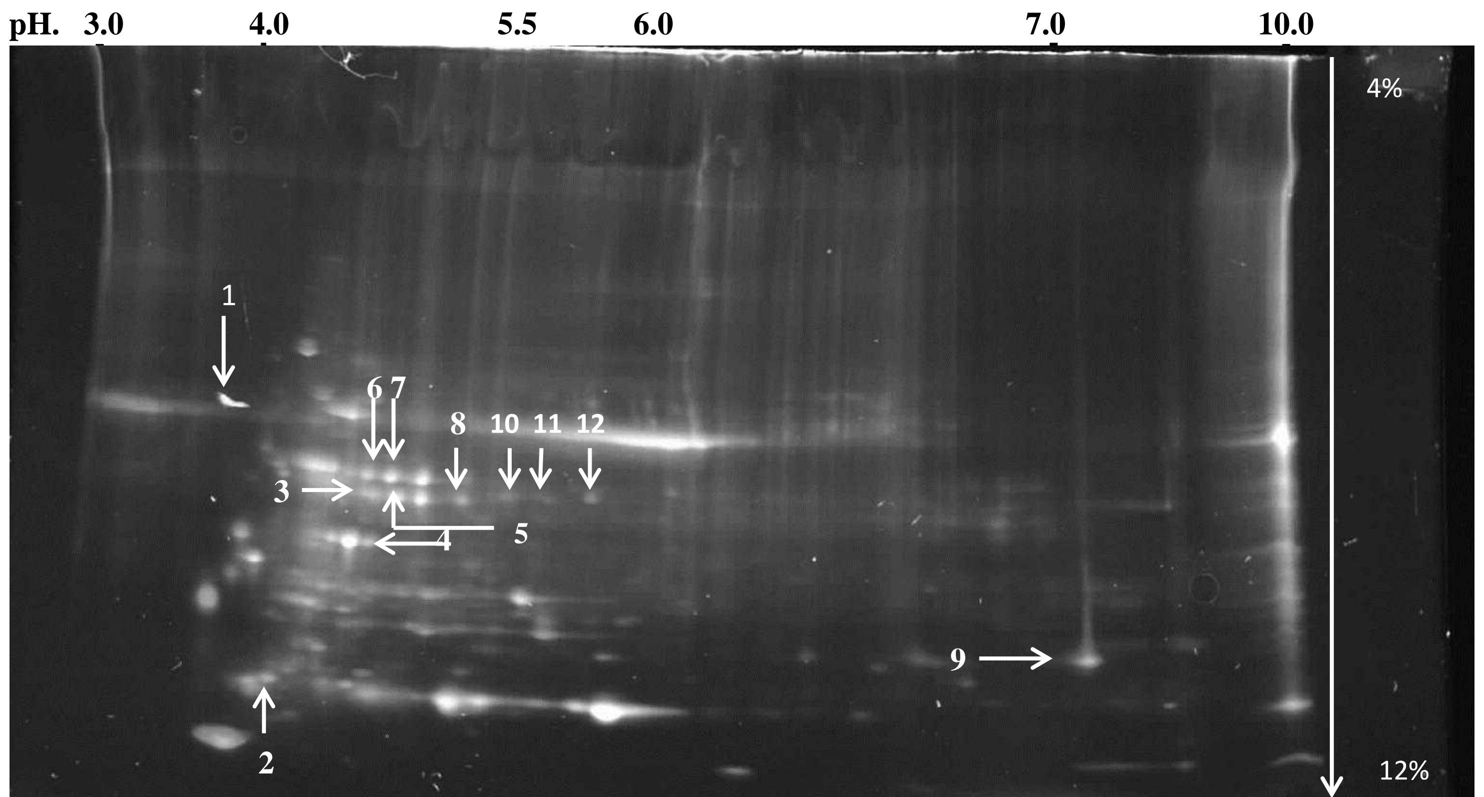


Figure 4.8: [B2] – Representative 2D gel of the proteome of Tugela Dn plants infested with RWA-SA2 (3hrs post infestation). Arrows indicate the spots that were identified as present in the Tugela Dn infested plants but not infested Tugela plants. The numbers 1 – 12 correlate with the numbers in the numbers in table 4.1 which list the identified proteins

Table 4.1: Proteins identified from the wheat leaf proteome (Tugela Dn) 3hrs post aphid infestation.

N	Putative protein ID	Acc. No.	%Cov(95)	Theoretical pI/Molecular weight (Dalton)	Function	References
1	RuBisCO large subunit-binding protein subunit α	P08823	32.9	4.03/57520.59	RuBisCO catalyses 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
2	Ribulose bisphosphate carboxylase small chain clone 512	P07398	20.4	5.84/ 13055.05		
3	Fructose-1,6-bisphosphatase	P09195	7.4	5.14/44218.07	This protein is involved in the pathway Calvin cycle, which is part of carbohydrate biosynthesis	Raines *et al.*, 1988 Lloyd *et al.*, 1991
4	Photosystem II D2 protein	Q36814	46.3	5.34/39470.38	Photosystem II (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. The D1/D2 (PsbA/PsbA) reaction centre 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-Bowdoin *et al.*, 1988 Ogihara *et al.*, 2000
4	Photosystem II protein D1	P12463	42.9	5.21/ 38920.62		
4	Dehydrin COR410	P46524	9.1	5.19/28183.26	largely function in plant response and adaptation to abiotic stresses	Hanin *et al.*, 2011
5	Phosphoribulokinase	P26302	16.4	5.72/45141.39	This protein is involved in the pathway Calvin cycle, which is part of carbohydrate biosynthesis. Catalytic activity: ATP + D-ribulose 5-phosphate = ADP + D-ribulose 1, 5-bisphosphate.	Raines *et al.*, 1989 Lloyd *et al.*, 1991
6	ATP synthase subunit β	P20858	20.1	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
7	S-adenosylmethionine synthase	B0LXMO	4	5.55/43179.90	Catalyses the formation of S-adenosylmethionine from methionine and ATP.	Wang *et al.*, 2008

					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	
8	**Phosphoglycerate kinase**	**P12783**	**4**	**6.57/49839.53**	**This protein is involved in the pathway Calvin cycle, which is part of carbohydrate biosynthesis** **Catalytic activity: ATP + 3-phospho-D-glycerate = ADP + 3-phospho-D-glyceroyl phosphate.**	**Longstaff *et al.*, 1989** **Jones *et al.*, 1995**
8	**Ribulose bisphosphate carboxylase large chain**	**P11383**	**31**	**6.22/52851.13**	**RuBisCO catalyses 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**
9	**Ribulose bisphosphate carboxylase small chain PW9**	**P26667**	**14.2**	**8.52/19454.45**		
10	**ATP synthase subunit α**	**P12112**	**18**	**5.70/55264.20**	**Produces ATP from ADP in the presence of a proton gradient across the membrane. The catalytic sites are hosted primarily by the beta subunits.**	**Schulte *et al.*, 1989**
11	**Ribulose bisphosphate carboxylase small chain PWS4.3**	**P00871**	**20.4**	**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 (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**
12	**Sedoheptulose-1,7-bisphosphatase**	**P46285**	**41**	**6.04/42060.82**	**This protein is involved in the pathway Calvin cycle, which is part of carbohydrate biosynthesis** **Catalytic activity: Sedoheptulose 1,7-bisphosphate + H_2O = sedoheptulose 7-phosphate + phosphate**	**Raines *et al.*, 1992** **Miles *et al.*, 1993**

4.3.4.2) 6-hours Tugela and Tugela Dn wheat responses to RWA-SA2 phloem feeding

When the proteomes of Tugela and Tugela Dn were evaluated after 6 hours post infestation, only 3 proteins were exclusively expressed in either infested Tugela Dn (Figure 4.9 [B3]) or infested Tugela (Figure 4.10 [B4]), indicating a significant drop in differential expressed unique proteins compared to the 3 hours post infestation. Two exclusively expressed protein spots [13 and 14] were seen for Tugela Dn, both identified as ATP synthase subunit β [pI = 5.06; MW = 53857.48Da] (Table 4.2. It would appear that these are either isoforms of one another or represent possible different phosphorylated states of the protein. Phosphoribulokinase and Phosphoglycerate kinase appeared to have co-eluted with this protein. The effect of RWA-SA2 phloem feeding on Tugela Dn, 6hrs post infestation also revealed that the integrity of the Tugela Dn proteome appears to be still intact. The single unique protein expressed in Tugela at 6hrs post RWA-SA2 infestation was identified as Photosystem II D1 and D2 protein, possibly indicating the plants response to repair a damaged photosynthetic system.

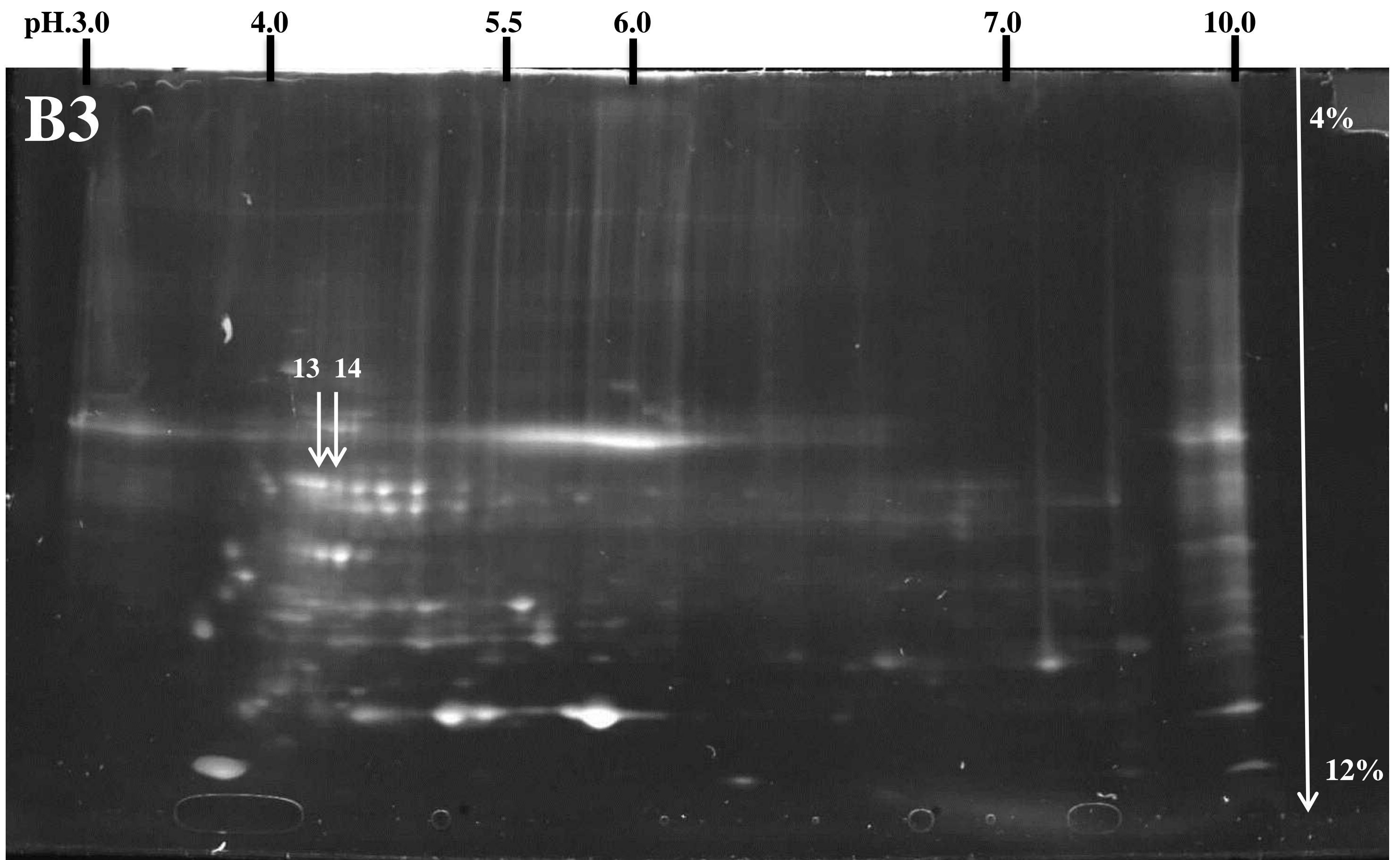


Figure 4.9: [B3] – Representative 2D gel of the proteome of Tugela Dn plants infested with RWA-SA2 (6hrs post infestation). Proteins putatively identified as indicated by the arrows and numbers correlate with the numbers in Table 4.2 which lists the identified proteins

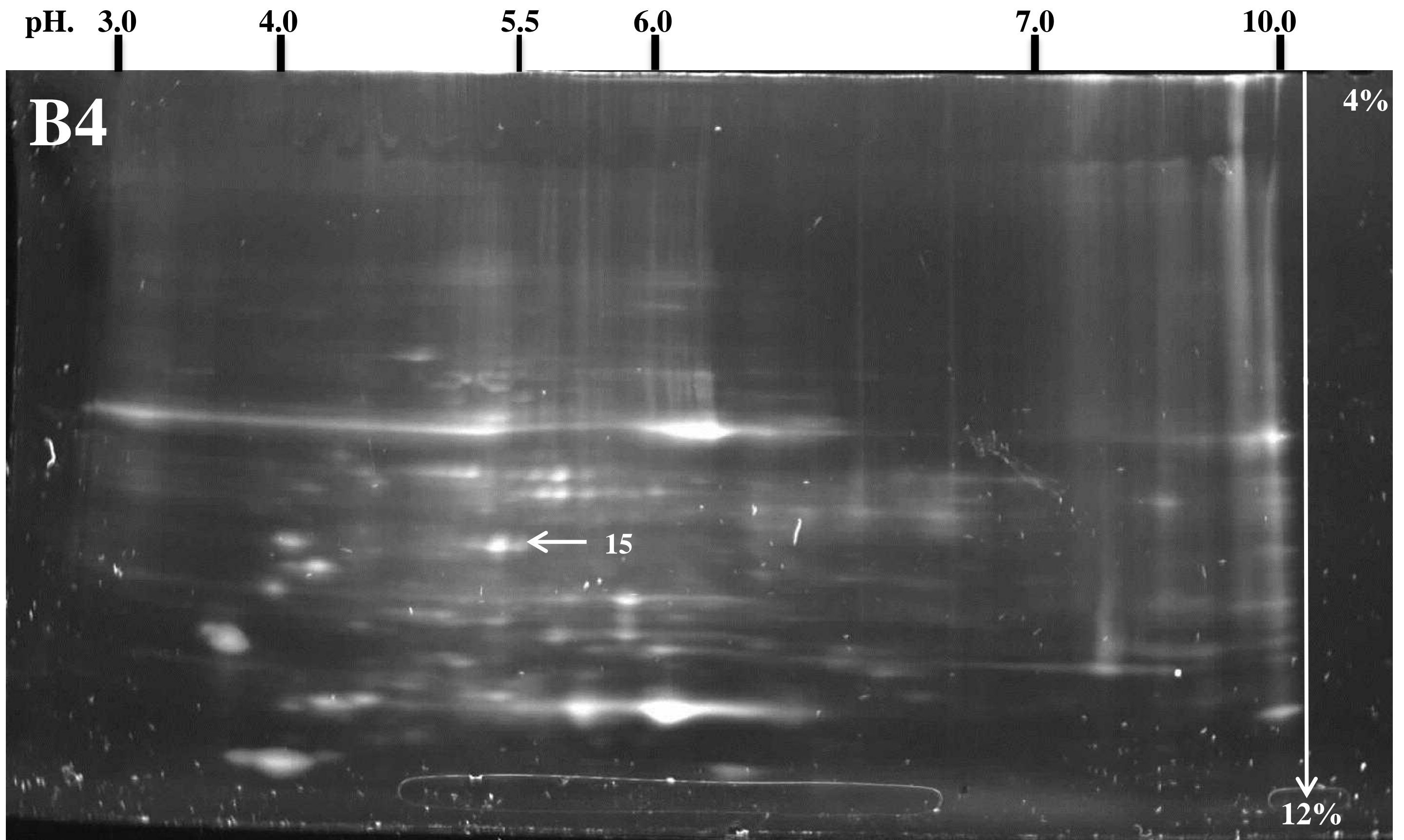


Figure 4.10: [B4] – Representative 2D gel of the proteome of Tugela susceptible stressed with RWA-SA2 (6hrs post infestation). Proteins putatively identified as indicated by the arrows and numbers correlate with the numbers documented in Table 4.2 which list the identified proteins.

Table 4.2: Proteins identified from the wheat leaf proteome of Tugela susceptible and Tugela Dn, 6hrs post aphid infestation.

N	Putative protein ID	Acc. No.	%Cov(95)	Theoretical pI/Molecular weight (Dalton)	Function	References
13	ATP synthase subunit β	P20858	11.5	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
14	ATP synthase subunit β	P20858	16.5	5.06/53857.48		
15	Photosystem II D2 protein	Q36814	30.6	5.34/39470.38	Photosystem II (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. The D1/D2 (PsbA/PsbA) reaction centre 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-Bowdoin *et al.*, 1988 Ogihara *et al.*, 2000
15	Photosystem II protein D1	P12463	34.4	5.21/ 38920.62		

4.3.4.3) 7 - Days Tugela and Tugela Dn wheat responses to RWA-SA2 phloem feeding

At the 7th day post RWA-SA2 infestation, both proteomes of Tugela and Tugela Dn clearly showed signs that their proteomes were compromised (Figure 4.11 [B5]-Tugela Dn and Figure 4.12 [B6]-Tugela) with several proteins in the pI range of 4 – 7 and 35 – 55kDa clearly absent in both the Tugela Dn infested and Tugela infested proteome. When compared to the proteomes at 3hrs and 6hrs, proteins that were identified as being exclusively expressed after day 7 post infestation were indicated as protein spot number 16 & 18 [Figure 4.11 B5 – Tugela Dn –infested] and spots 17 & 19 [Figure 4.12 B6 – Tugela –infested]. These proteins appeared to be low molecular weight proteins and were identified as photosynthesis related, structural support and kinases. The proteins in Tugela Dn day 7 post infestation were identified as Ribulose bisphosphate carboxylase small chain clone 512 (pI=5.84; MW= 13055.05Da) (spot 16) and Chlorophyll a-b binding protein (pI=5.67, MW=28264.35Da) (spot 18). In Tugela, the exclusively expressed proteins were identified as 2-Cys peroxiredoxin BAS1 (pI= 5.7; MW=23326Da) (spots 17 and 19) with two other proteins (Protein-L-isoaspartate O-methyltransferase and Chlorophyll a-b binding protein) appearing to have co-eluted with it.

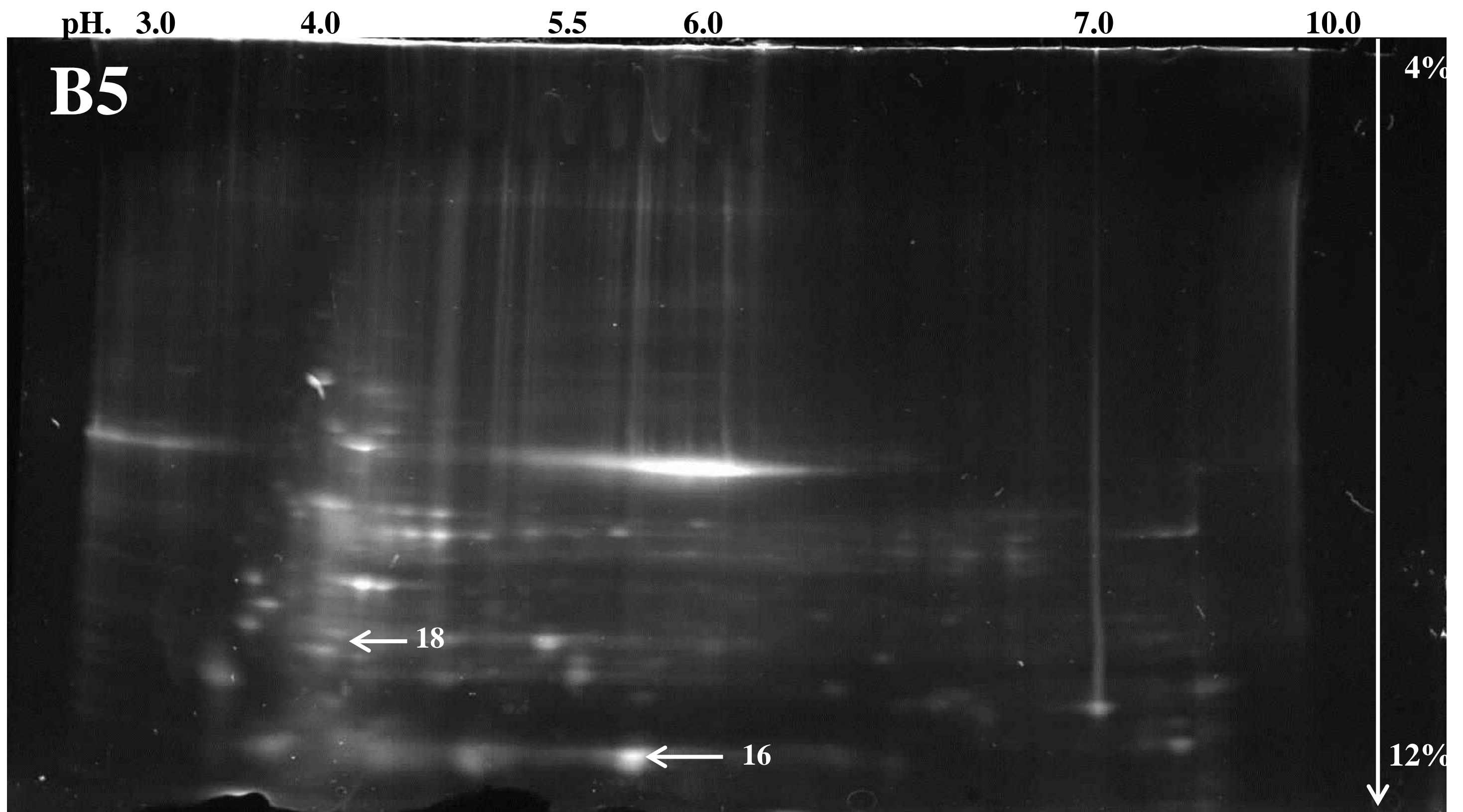


Figure 4.11: Representative 2D gel of the proteome of Tugela Dn infested with RWA-SA2 (Day 7 post infestation). Proteins putatively identified as indicated by the arrows and numbers are clearly documented in Table 4.3.

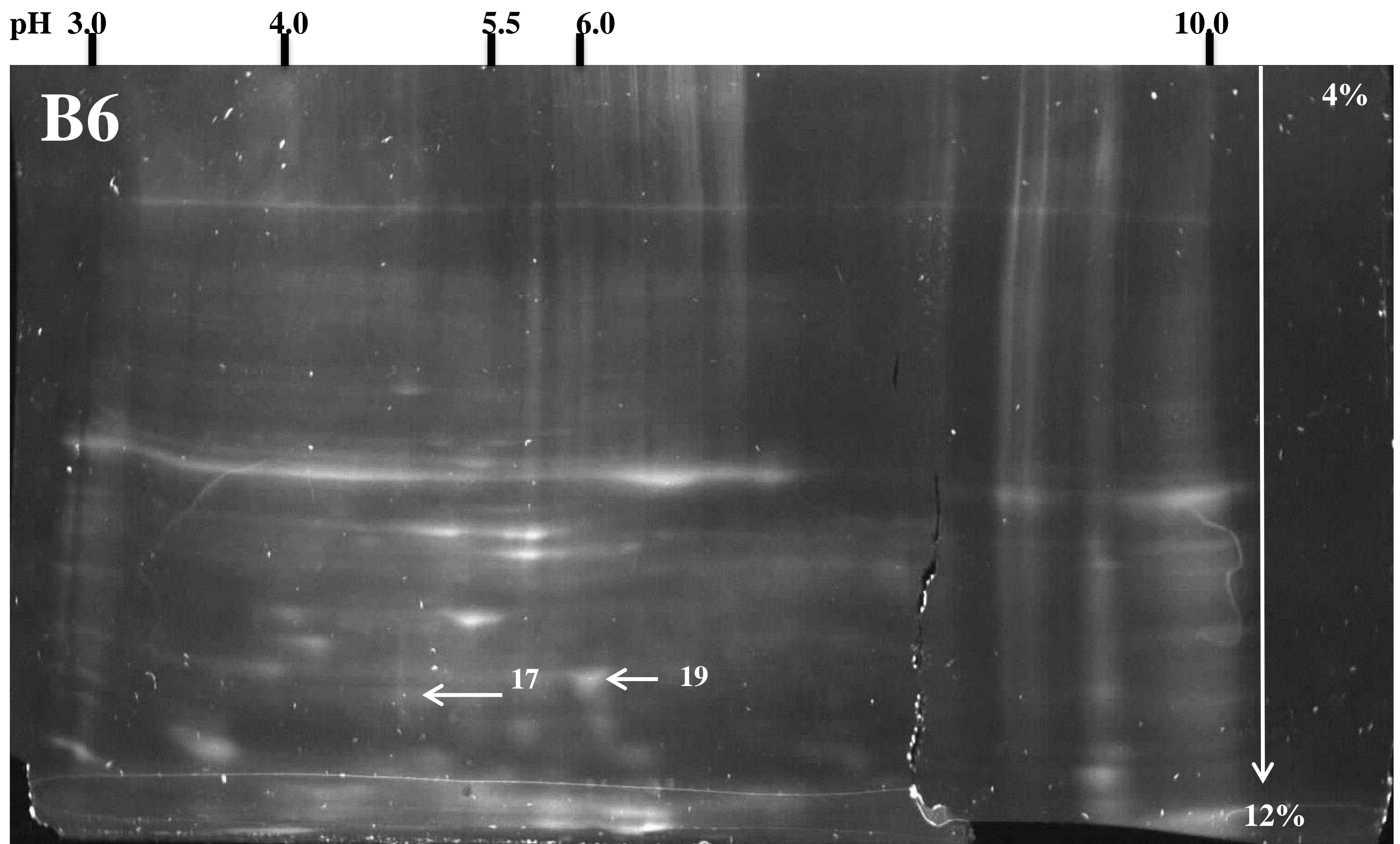


Figure 4.12: [B6] – Representative 2D gel of the proteome of Tugela susceptible stressed with RWA-SA2 (Day 7 post infestation). Proteins putatively identified as indicated by the arrows and numbers are clearly documented in Table 4.3.

Table 4. 3: Proteins identified from the wheat leaf proteome of Tugela susceptible and Tugela Dn, Day 7 post aphid infestation.

N	Putative protein ID	Acc. No.	%Cov(95)	Theoretical pI/Molecular weight (Dalton)	Function	References
16	Ribulose bisphosphate carboxylase small chain clone 512	P07398	89.4	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 clone 512	P07398	56	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).	
17	2-Cys peroxiredoxin BAS1	P80602	46.7	5.71/23326.68	May be an antioxidant enzyme particularly in the developing shoot and photosynthesizing leaf	Tsunoyama *et al.*, 1996 Tsunoyama & Toyoshima, 1997
18	Chlorophyll a-b binding protein	P04784	16.2	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
19	2-Cys peroxiredoxin BAS1	P80602	4.3	5.71/23326.68	May be an antioxidant enzyme particularly in the developing shoot and photosynthesizing leaf	Tsunoyama *et al.*, 1996 Tsunoyama & Toyoshima, 1997
19	Chlorophyll a-b binding protein	P04784	12.8	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
19	Protein-L-isoaspartate O-methyltransferase	Q43209	4.3	4.90/24708.02	Recognizes and catalyzes the repair of damaged L-isoaspartyl and D-aspartatyl groups in proteins	Johnson *et al.*, 1993

The function of the various unique expressed proteins were analysed for the three timeframes, 3hrs, 6hrs and 7 days and proteins with similar functions totalled up to provide a possible indication of functional differences between the response of Tugela Dn and Tugela after infestation with RWA-SA2 (Figure 4.13).

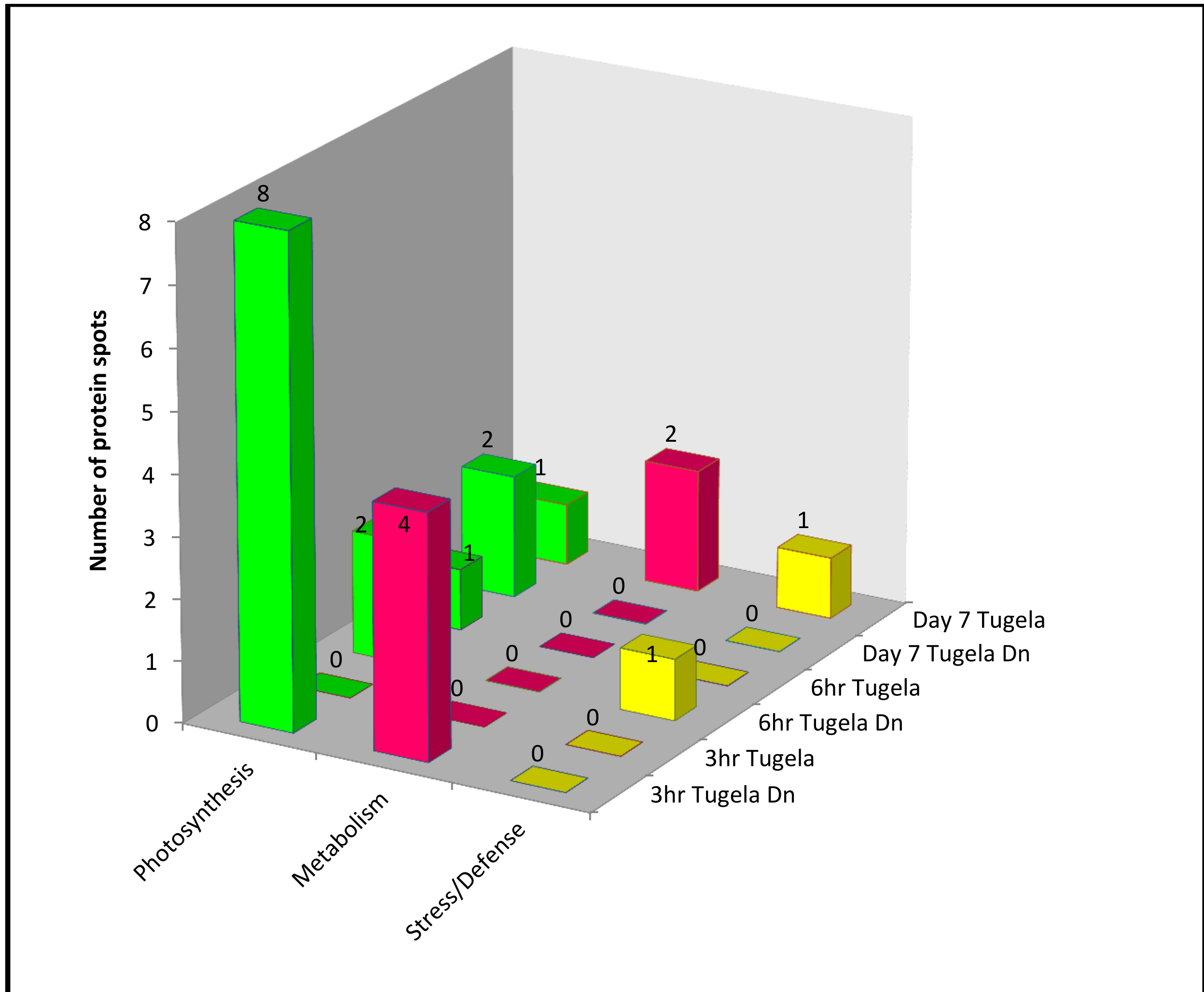


Figure 4.13: Distribution and assignment of putatively identified proteins after RWA-SA2 infestation on infested Tugela Dn and Tugela at the three indicated harvest times. Exclusively expressed proteins per timeframe were taken as the total number for each stress condition in order to construct the histogram. Different classes of proteins were grouped according to function as represented by the different colours.

The most significant differences between the response of Tugela Dn and Tugela to RWA-SA2 was observed at 3hrs post infestation, with 66% of the exclusively expressed proteins in Tugela Dn being related to photosynthesis while 33% were related to metabolism. At 6hrs and 7 days, proteins related to photosynthesis were still expressed at a higher level in Tugela Dn than in Tugela, while additional defense or stress signalling proteins were upregulated in Tugela Dn at 6hrs and 7 days but not in Tugela.

4.3.5) Identification of pathways involved in the resistance response to RWA-SA2 phloem feeding

4.3.5.1) Proteins/enzymes involved in photosynthesis related pathways

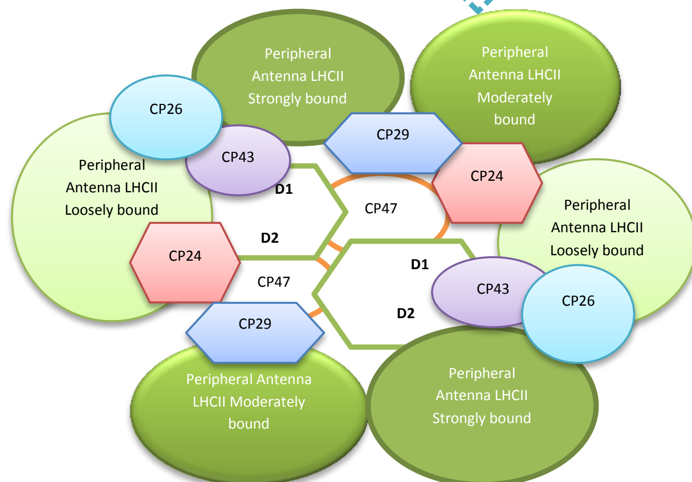


Figure 4.14: Model of the electron transport chain taken from http://www.metacyc.org/. Proteins/enzymes identified in the response in Tugela Dn to RWA-SA2 and their involvment in the electron trasport: ATP synthase subunit α, ATP synthase subunit β, Chlorophyll a-b binding protein, Photosystem II D2 protein and Photosystem II protein D1.

Left (Insert): Constructed image of a photosystem II- LHCII supercomplex adapted from van Grodell, 2004 and Boekema, 1999. D_1 and D_2 coordinate the photoactive reaction centre P680. This is where charge separation occurs.

The ability to photosynthesize is a distinctive characteristic that is unique to algae, plants as well as photoautotrophic bacteria. Photosynthesis is composed of the light reactions and the dark reactions. The light reactions take place in both photosystem I and photosystem II, where energy from the light is captured by Chlorophyll a and b pigments – which are located in the thylakoid membrane of the chloroplast - is used to energize the transfer of electrons from water, through a series of electron donors and acceptors, to the final acceptor NADP+, which is reduced to NADPH (Blackenship, 1992). The NADPH generated by the light reactions is used for sugar synthesis in the dark reactions. The light reactions also generate a proton motive force across the thylakoid membrane, and the proton gradient is used to synthesize ATP through the ATP synthase complex.

4.3.5.1.1) Chlorophyll a-b binding protein

Chlorophyll a-b binding protein has been implicated in the light-harvesting complex (LHC) and functions as a light receptor in capturing and delivering excitation energy to photosystems with which it is closely associated (Lamppa *et al.*, 1985). Up-regulation of chlorophyll a-b binding protein in Tugela Dn 7days after phloem feeding could possibly signal that this delayed response by the plant is a possible indication that RWA-SA2 has imposed on the photosynthetic apparatus of the plant so that the plant can keep pace of its metabolism.

4.3.5.1.2) Photosystem II protein D1 and Photosystem II D2 protein

According to Nanba and Satoh (1987), Photosystem II protein D1 and Photosystem II D2 protein are crucial proteins that constitute the photosystem II reaction centre complex together with other proteins. This reaction centre plays a pivotal role in capturing photons of light. Plastoquinone oxidoreductase uses the 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. The D1/D2 (PsbA/PsbA) reaction centre heterodimer binds P680, the primary electron donor of PSII as well as several subsequent electron acceptors.

Photosystem II D1 and D2 proteins are needed for assembly of a stable PSII complex (Figure 4.14 [Insert]) (Ogihara *et al.,* 2000). Photosystem II protein D1 and Photosystem II D2 protein was down-regulated in Tugela 3hrs post RWA-SA2 infestation and then up-regulated in Tugela 6hrs post infestation which possibly indicates the plants attempt to restore normal photosynthetic function.

4.3.5.1.3) ATP Synthase

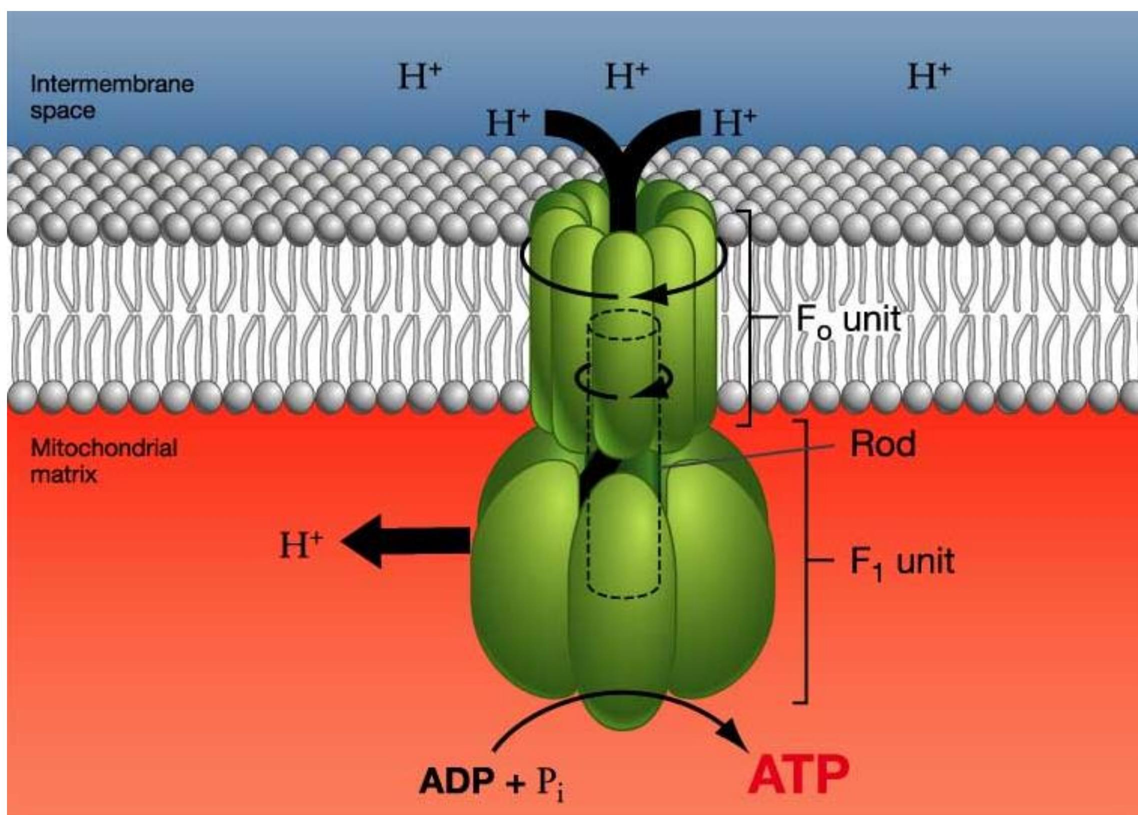


Figure 4.15: Structure of ATP synthase taken from (http://www.metacyc.org/). ATP is generated as H^+ moves down its concentration gradient through ATP synthase

ATP synthase catalyses the synthesis of ATP from ADP and inorganic phosphate (Pi) in the presence of a proton gradient across the membrane. 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 (Howe *et al.*, 1985 and Ogihara *et al.*, 2000) .

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. Studies have also shown that ATP is known to induce the expression of genes involved in the biosynthesis of Jasmonic acid and ethylene which important pathways in plant defense (Choi *et al.*, 2014). JA and ET have been proposed to activate specific genes that promote wound healing (Asahina *et al.*, 2011). ATP synthase has also been found to be essential and an integral component of photosynthesis in which it plays a key role in the channelling of proteins across the membrane (Maiwald *et al.*, 2003) Upregulation of ATP synthase in Tugela Dn 3hrs and 6hrs post RWA-SA2 infestation is indicative of the plant counteracting RWA aphid probing and maintaining normal cellular functioning and photosynthetic activity to proceed.

4.3.5.1.4) 2-Cys peroxiredoxin

2-Cys peroxiredoxin BAS1 has been found to be a possible antioxidant enzyme particularly in the photosynthesizing machinery of the plant. Its upregulation in Tugela 7 days post RWA-SA2 infestation could suggest that the enzyme serves as an antioxidant as the plants photosynthetic machinery has already succumbed to RWA feeding. This could possible lead to irreversible photo-inhibition (Kyle, 1987), resulting in PSII D1 and D2 proteins being a possible site of *D. noxia* damage. Ohad *et al.* (1990) further emphasize that photo-inhibition caused by over-reduction on the photon acceptor side will in turn lead to an impaired donor side. With this electron flow being hampered, electrons may follow an alternative route that might increase Reactive Oxygen Species (ROS) production (Vas *et al.*, 1992) which could lead to chloroplast dilapidation and peroxidation of thylakoid lipids if these ROS molecules react with chloroplast macromolecules (Mishra and Singhal, 1992). ROS production together with the combined action of nitrous oxide (NO) triggers the HR. Studies conducted by Mohase and Van der Westhuizen (2002) shows that the HR can also cause salicylic acid (SA) and benzoic acid (BA) production which travels from the infection site to nearby tissues as a secondary defense mechanism. SA is associated with triggering the expression of a set of defense gene families encoding certain pathogenesis related (PR) proteins. PR proteins include lytic enzymes i.e. chitinases, glucanases that degrade fungal and bacterial cell walls and lectin-like, thionin-like and proteinaseinhibitor-like antifungal proteins (Van Wees *et al*., 2000).

SA can also trigger systemic expression of PR proteins in the other parts of the plant. This trigger leads to a long-lasting and broad-spectrum resistance response called the systemic acquired resistance (SAR) that is non-specific (Metraux *et al*., 1990).

4.3.5.2) Proteins/enzymes involved in metabolism related pathways

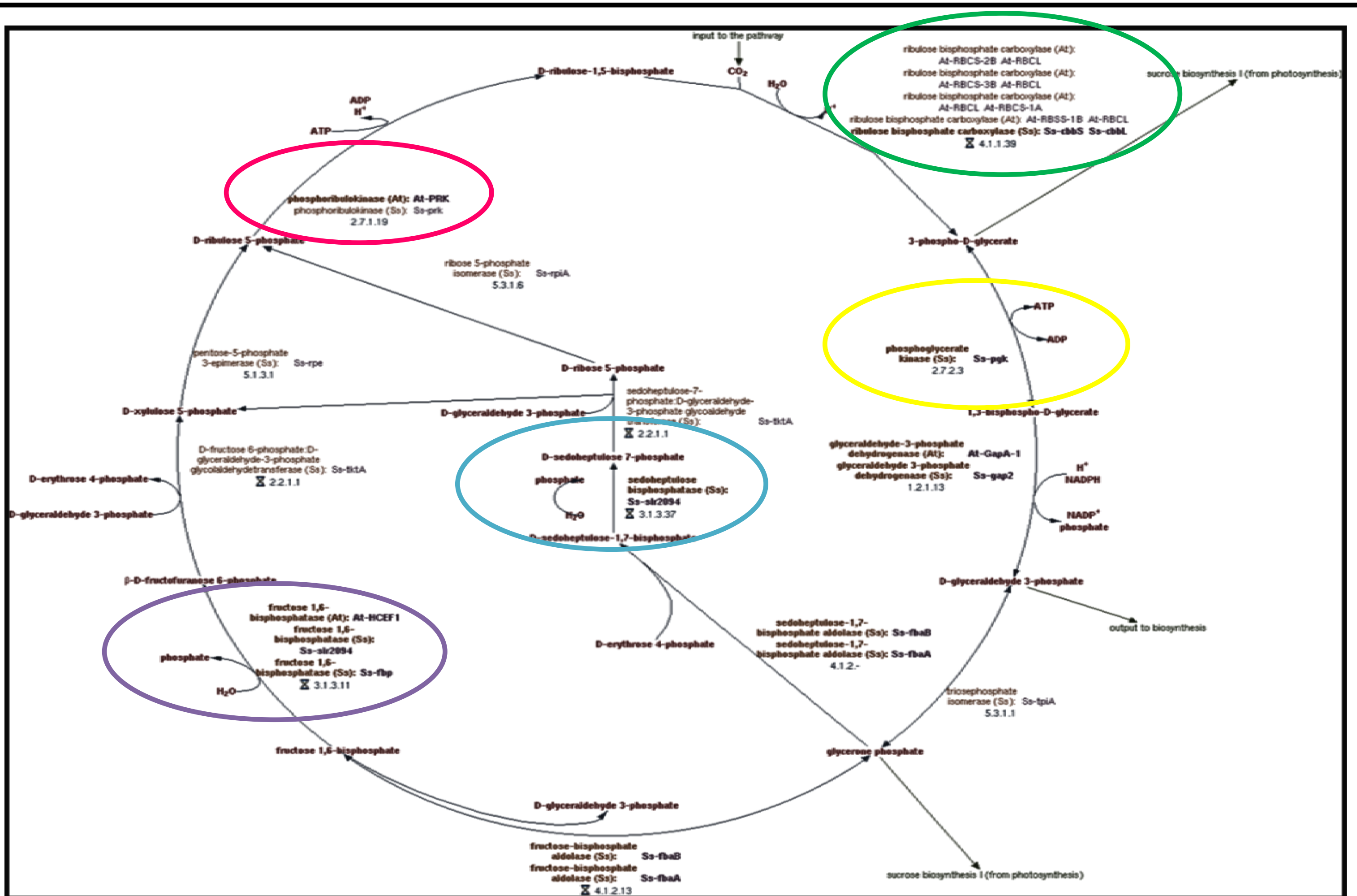


Figure 4.16: Shows the involvement of the putatively identified proteins/enzymes and the reactions they catalyse in the Calvin cycle. Figure taken from http://www.metacyc.org/

- (green) **RuBisCO large subunit-binding protein subunit α: 2 3-phospho-D-glycerate + 2 H+ ↔ D-ribulose-1, 5-bisphosphate + CO_2 + H_2O**
- (yellow) **Phosphoglycerate kinase: 3-phospho-D-glycerate + ATP ↔ 1, 3-bisphospho-D-glycerate + ADP**
- (blue) **Sedoheptulose-1, 7-bisphosphatase: D-sedoheptulose-1, 7-bisphosphate + H_2O → D-sedoheptulose 7-phosphate + phosphate**
- (purple) **Fructose-1, 6-bisphosphatase: Fructose 1, 6-bisphosphate + H_2O → β-D-fructofuranose 6-phosphate + phosphate**
- (pink) **Phosphoribulokinase: D-ribulose 5-phosphate + ATP ↔ D-ribulose-1, 5-bisphosphate + ADP + H+**

The Calvin cycle is the major CO_2 fixation pathway, found in all in green plants and many autotrophic bacteria. During this cycle, one CO_2 molecule at a time is added to the acceptor molecule D-ribulose-1, 5-bisphosphate, producing two molecules of 3-phospho-D-glycerate this is then passed through a cascade of reactions, regenerating the molecule D-ribulose-1, 5-bisphosphate.

The Calvin cycle is divided into three stages: fixation, reduction and regeneration. Proteins/enzymes identified in this pathway in response to aphid feeding include Phosphoribulose kinase, phosphoglycerate kinase, sedoheptulose-1,7-bisphophatase and ribulose bisphosphate carboxylase. In the first stage of the Calvin cycle the reduction carboxylation of D-ribulose-1,5-bisphosphate (RuBP) is catalysed by the enzyme ribulose bisphosphate carboxylase (RuBiSCO) forming two molecules of 3-phospo-D-glycerate. The second step involves these two molecules that are phosphorylated to 1,3-bisphospho-D-glycerate and then again dephosphorylated to D-glyceraldehyde 3-phosphate. In three cycles of this 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 involves regenerating 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 latter 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, which then becomes 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, restoring the key CO_2-acceptor molecule (Caspi *et al.*, 2014). Phosphoribulose kinase, phosphoglycerate kinase, sedoheptulose-1,7-bisphophatase and ribulose bisphosphate carboxylase were down-regulated in Tugela 3hrs post RWA-SA2 infestation possibly indicating that the plant‘s primary metabolism was damaged by RWA feeding and led to possible leakage/loss of metabolites. These proteins/ enzymes were constitutively up-regulated in Tugela Dn in response to RWA-SA2 infestation indicating that the resistant cultivar is able to cope better with the feeding/probing damage caused by RWA. However, plants also have to take care in controlling the carbon flux between this and competing pathways to make sure that the Calvin cycle can proceed due to rate limiting steps that do exist (Caspi *et al.*, 2014). To achieve this, the catalytic activities of certain enzymes within the cycle are highly regulated including sedoheptulose-1, 7-bisphosphatase and fructose-1,6-bisphosphatase 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, fructose-1,6-bisphosphatase functions at the branch point between the regenerative phase of the Calvin cycle and starch biosynthesis, and sedoheptulose-1, 7-bisphosphatase and fructose-1,6-bisphosphatase catalyse irreversible reactions (KoBmann *et al.*, 1994). Lefebvre *et al.* (2005) reported that increase in sedoheptulose-1, 7-bisphosphatase stimulates photosynthesis and growth rate in Arabidopsis.

4.3.5.3) Proteins/enzymes involved in stress related pathways

4.3.5.3.1) Dehydrins

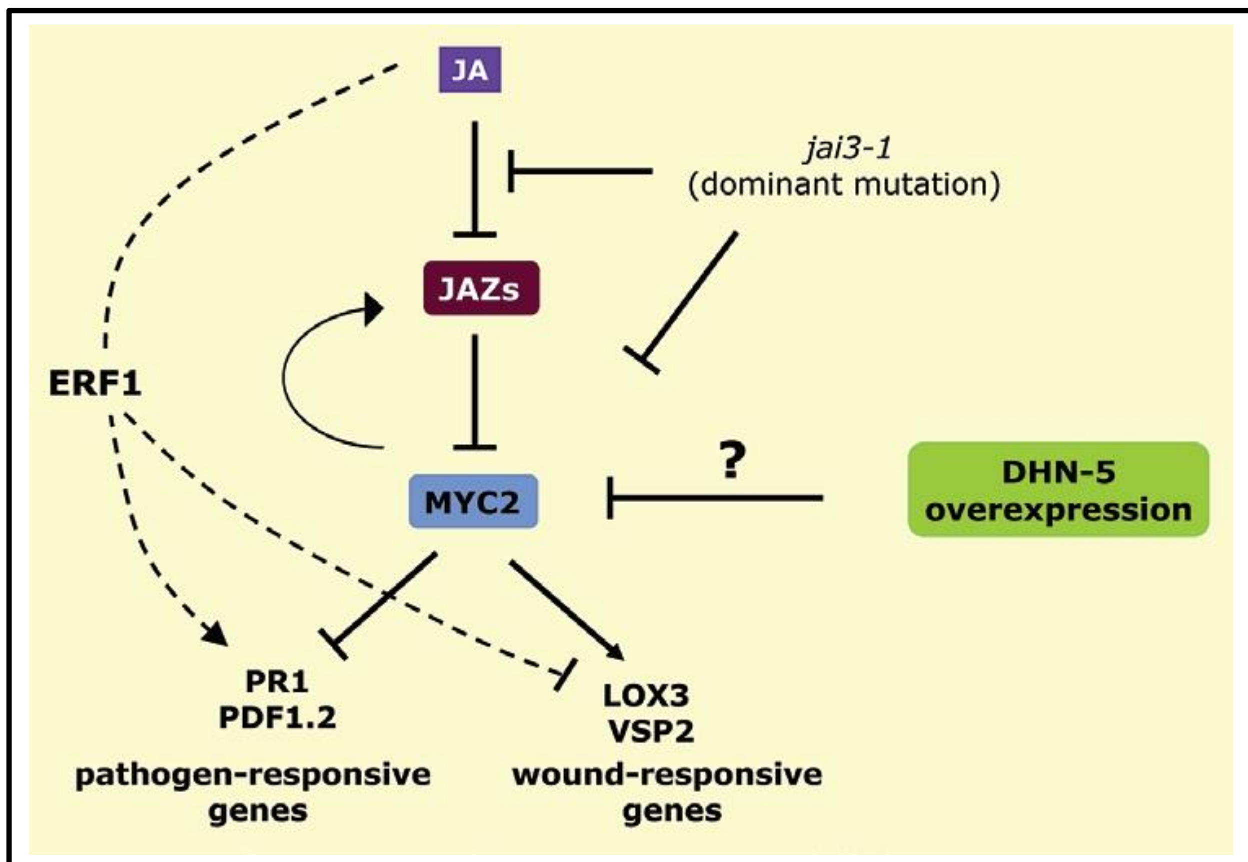


Figure 4.17: possible involvement of Dehydrin in biotic stress tolerance. Figure taken from Hanin *et al.*, 2011.

Dehydrins (DHNs) belong to a group of proteins called the Late Embryogenesis Abundant or (group 2 LEA proteins) and they largely function in plant response and adaptation to abiotic stresses. However, it has been reported that wounding induced the expression of specific Dehydrins.

Wounding is a common biotic stress which is usually exerted by insects or other herbivorous species and is also regarded as dehydration stress because of its association with cellular damage that leads to water loss. Furthermore, wounding is also known to increase the expression of sucrose transporters (Sakr *et al.*, 1993; Sakr *et al.*, 1997; Meyer *et al.*, 2004; Kempema *et al.*, 2007). Plants need to compensate for this osmotic stress and nutritive loss exerted on them in response to aphid feeding. To this effect, they use sucrose in the presence of sucrose transporters to drive it (Sauer, 2007) and to replenish any nutritive loss that may have occurred. In spite of its contribution in enhancing osmotic stress tolerance, it has recently been reported that Dehydrins affects the expression of not only genes involved in abiotic stress but also implicated in defense responses. Interestingly, the authors also found that Dehydrins interferes with Jasmonic Acid (JA) signalling. JA is an imperative signalling molecule in defense mechanisms against pathogens.

4.3.5.3.2) Protein-L-isoaspartate O-methyltransferase

Protein L-isoaspartyl methyltransferase (PIMT) has been found to combat protein misfolding resulting from L-isoaspartyl formation by catalyzing the conversion of abnormal L-isoaspartyl residues to their normal L-aspartyl forms (Johnson *et al.*, 1993). In this way, the enzyme contributes to longevity and survival in bacterial and animal kingdoms. It's role during plant stress still remains undefined. A study conducted on Arabidopsis thaliana lines exhibiting altered expression of PIMT1, one of the two genes encoding the PIMT enzyme in Arabidopsis has shown that overaccumulation of PIMT1 reduced the build-up of L-isoaspartyl residues in seed proteins and increased both seed longevity and germination vigour. On the contrary, reduced PIMT1 accumulation was associated with an increase in the accumulation of L-isoaspartyl residues in the proteome of freshly harvested dry mature seeds, thus leading to amplified sensitivity to aging treatments and loss of seed vigour under stressful germination conditions. Thus the PIMT enzyme is likely to work as a unit with other anti-aging pathways to actively eradicate deleterious protein products, enabling successful seedling establishment and strengthening plant proliferation in natural environments (Oge' *et al.*, 2008). One may therefore argue that the upregulation of the PIMT enzyme in Tugela day 7 post RWA-SA2 infestation is an attempt by the plant to repair amino acid residues for normal cellular functioning and other metabolic processes such as protein synthesis to proceed even though the plant has already succumbed to RWA probing.

4.4) Discussion and conclusions

The differences in the temporal and composition of differentially expressed proteins clearly indicate that Tugela and Tugela Dn wheat cultivars activate a range of different responses to RWA-SA2 phloem feeding. Bilgin *et al.* (2010) has also provided evidence that regardless of the type of biotic stress on a plant, the response appears to be generally a down-regulation of photosynthetic related genes more so in susceptible plants. Their study has also shown that certain host plants tend to down-regulate these photosynthetic related genes as an adaptive response to biotic attack but does not necessarily result in the hosts' ability to function normally.

The findings presented here appear to be in accordance with the findings of Botha *et al.* (2006) indicating that the wheat cultivar that contains the *Dn* resistance gene, Tugela Dn, experiences a potential delayed defense response in response to RWA-SA2 feeding. Furthermore, RWA-SA2 has caused more physical damage to Tugela plants than Tugela Dn plants which appears to be in line with the findings of Tolmay *et al.* (2007) and Walton and Botha (2008) who reported that RWA-SA2 not only bred faster but also caused more damage to wheat lines than did RWA-SA1 and appeared to be unaffected by the *Dn*1 resistance gene.

Results obtained from this study also indicate that majority of the putatively identified proteins are involved in photosynthesis and metabolism. Botha *et al.* (2006) have found similar findings with their DNA microarray data in which they have identified transcripts which encode for proteins functioning in cell wall degradation, cell maintenance, defense, oxidative burst, photosynthesis and energy metabolism. Their study also confirmed that majority of these protein encoding genes were observed within the first 24 hours after infestation. Van der Westhuizen and Botha (1993) also reported that the protein profile of cereal plants begin to change within 48 hours, when they are infested with the RWA. In their study, they compared responses of Tugela (susceptible) and TugelaDN (resistant) infested with RWA (South African Biotype one). Their results showed an increase in the number of proteins in the resistant cultivar and a decrease in the susceptible cultivar. Although the aphids prefer to feed at the bases of the leaves, the profiles of all the different parts of the resistant leaf were similar, indicating a global expression of "protective components".

The putative resistance proteins identified in this study, are involved in photosynthetic electron transport chain and proteins belonging or associated to photosystem I & II

complexes and the Calvin cycle metabolic pathway. Botha *et al.* (2006) also investigated whether photosynthetic transcriptional regulation is a contributing factor for tolerance to *D. noxia* and found that the chloroplast machinery maintenance is one of the determining factors in permitting resistant plants to overcome the stress imposed by RWA feeding. They found that ATP-synthase plays a significant role in this regard through the provision of energy during cell maintenance and may intensify the plants' stress tolerance by keeping the plants' photosynthetic apparatus intact. The down-regulation of RuBisCO (RuBP) in Tugela susceptible could possibly suggest that the rate of incorporation and/or exportation of photosynthetic products decline, as a result, photosynthesis becomes restricted by "feedback inhibition" (Lambers *et al.*, 1998) leading to modifications of source-sink feedback signals (Peterson and Higley, 1993). In comparison, Tugela Dn had increased RuBP rates which suggest faster regeneration of RuBP. The data in this study also indicates that before the appearance of visible signs of aphid attack, the progression of phloem feeding mostly represses photosynthesis related proteins in leaf as well as those involved in metabolism.

It would appear that the pathways identified function in parallel to capitalize on more defense efforts rather than resistance against RWA-SA2 as these pathways seem to be interlinked. This is in line with the findings of Botha *et al.* (2006), in which they propose that the up-regulation of PSII components involved in increased photosynthetic activity could potentially provide a mechanism of passive resistance against *D. noxia* feeding. Heng-Moss *et al.* (2003) proposed that a suggested site for RWA feeding damage would be the Light Harvesting Complex II (LHC II). The results from this study support this proposal, as the proteins identified here play a pivotal role in the LHC II. This study therefore provides evidence that Tugela Dn, to an extent, counteracts deleterious effects of aphid (RWA-SA2) herbivory through up-regulation and faster regeneration of photosynthetic related molecule and does respond in a highly specific manner to infestation with RWA-SA2 by inducing unique pathways. It would also appear that RWA-SA2 overcomes the resistance response of Tugela Dn against RWA-SA1 at a much later stage. Feeding by RWA-SA2 triggers an onset of a defense response in Tugela Dn and hypothesize that susceptible plants are likely not capable of maintaining energy production systems that rely on efficient photosynthetic activity due to chlorophyll breakdown. Further research is therefore needed to investigate the potential use of photosynthetic proteins and pathways identified in this study as potential markers for identifying *D. noxia* resistant germplasms.

CHAPTER 5

General Discussion, Conclusion and Future Work

The need to investigate resistance mechanisms could possibly lead to a better understanding of wheat stress responses to aphids in general, facilitating the development of improved cultivars possessing resistance to the effects of RWA infestation (Moloi and Van der Westhuizen, 2005). It has previously been reported that Tugela Dn has a better chance of surviving the stress condition imposed by RWA-SA1 than Tugela due to a higher proline content and subsequent maintenance of chloroplast integrity and photosynthesis (Van der Westhuizen and Pretorius, 1995). In addition to this "adapted protective mechanism" of Tugela Dn, Ni and Quisenberry (1997) reported that RWAs prefer wheat lines with shorter trichomes. Lines with longer trichomes positioned along the leaf veins were less preferred although the trichome density was less. The leaf veins are the preferred feeding site of RWA and the trichomes hinder their probing. The discovery was supported by the finding of Bahlmann *et al*., (2003) that the resistant wheat line Tugela Dn has more trichomes on the leaf veins than other non-resistant lines.

An increase in intercellular chitinase and peroxidase activity was reported within 48hr of RWA-SA1 infestation of Tugela Dn (Van der Westhuizen *et al.*, 1998b). However, this study showed that the pathway of RWA-SA1 probing appeared to be unaffected by the induction of these enzymes. In an analogous study, Van der Westhuizen *et al.* (1998a) reported that RWA-SA1 infestation on Tugela Dn induced a substantial increase of ß-1,3-glucanase activity while Botha *et.al*. (1998) reported a large induction of chitinase activity intercellularly of infested Tugela Dn. Infestation of Tugela Dn with RWA-SA1 also induced the accumulation of salicylic acid and increases peroxidase activity while catalase activity was inhibited (Mohase and Van der Westhuizen, 2002). This study was therefore undertaken to see whether RWA-SA2 overcomes the resistance of Tugela Dn against RWA-SA1 by identifying exclusively expressed proteins.

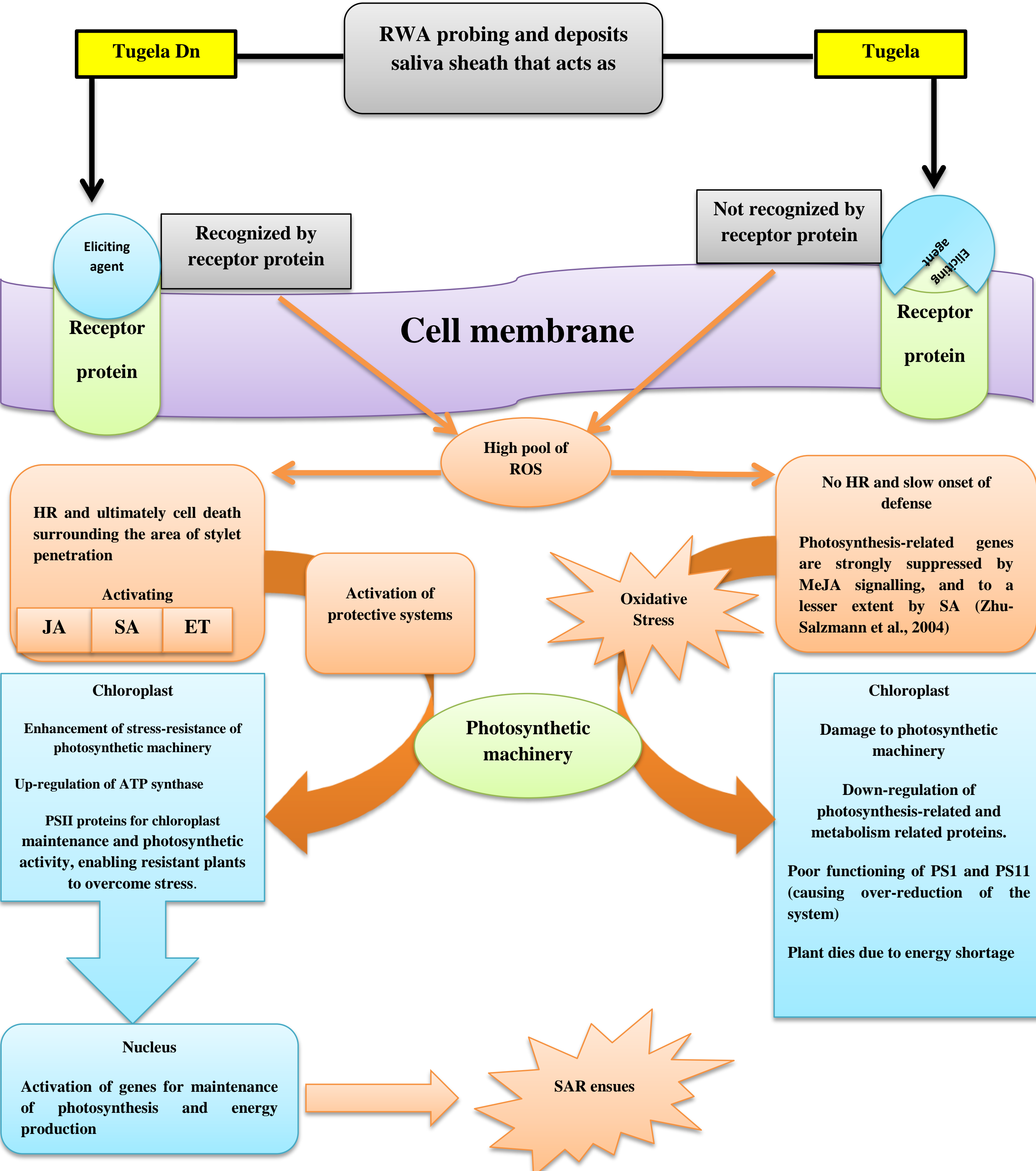
Extensive research efforts have been undertaken to characterize the mechanisms of resistance of the Russian Wheat Aphid as they are identified as a serious pest that has negatively impacted wheat as well as other cereal crops, (Burd and Elliott, 1996; Rafi *et al.*, 1996; Rafi *et al.*, 1997; Van der Westhuizen and Pretorius, 1995; Haile *et al.*, 1999). This study was undertaken to assess the effect the aphid feeding on the chlorophyll content in Tugela and Tugela Dn, by measuring variable chlorophyll fluorescence of the plants, a chlorosis is often seen in RWA infested plants. This was achieved through the use of the innovative Integrated Fluorometer and Photosynthesis system, which allows one to investigate photosynthetic reactions, light dependent reaction of a plant and plant photo-protection mechanisms (Baker,

2004). Chlorophyll a fluorescence has been found to have close correlation with light reactions in the photosynthetic tissues. Measuring chlorophyll a fluorescence is thus a non-invasive technique to study the light induced electron transfer in the multi-protein pigment complexes of photosynthesis (Schreiber, 2002). The results from this study indicated a decrease in F_m and F_v resulting in a decreased photochemical efficiency of photosystem II (PSII) (F_V/F_M) for Tugela (susceptible) whereas total chlorophyll concentrations was not significantly affected in Tugela Dn (resistant cultivar), are in line with those presented by Krause *et al.* (1982), Horton (1983) and Burd and Elliot (1996). It can therefore be concluded that Tugela Dn's resistance mechanism prevents the RWA's toxins from causing damage to the chloroplasts and the induction of expression of specific proteins in the chloroplast. The recognition of RWA specific proteins cause signal transduction to the nucleus of the plant cells and the induction of photosynthetic and metabolic related proteins. The photosynthesis machinery was thus maintained but the chlorophyll kinetic analysis indicates that the photosynthesis is compromised. The photosynthesis even had a greater decrease than observed in the susceptible Tugela plants. The reason for restricting photosynthesis might be to direct the energy to the formation of ROS rather than producing glucose for plant growth. By quickly producing ROS the plants stimulate the HR as an effective defense mechanism. Essential differences in the response to RWA feeding are thus observed in Tugela Dn compared to Tugela on a biological, physiological and protein level. This supports the hypothesis that photosynthesis does play a significant role in RWA resistance.

This study revealed that there appears to be commonality with some of the proteins/enzymes identified in response to stress imposed by RWA-SA1 feeding (Louw, 2007 [ATP Synthase α and β subunits, chlorophyll a-b binding protein and RuBiSCO), Nqumla, 2013 [ATP Synthase α and β subunits, fructose-1,6-bisphosphatase, RuBiSCO], and Njom, 2016 [ATP Synthase α and β subunits, chlorophyll a-b binding protein, RuBiSCO, Fructose-1,6-bisphosphatase, Phosphoribulokinase, Phosphoglycerate kinase, Ribulose bisphosphate carboxylase large chain, Ribulose bisphosphate carboxylase small chain PW9, Ribulose bisphosphate carboxylase small chain PWS4.3 and Sedoheptulose-1,7-bisphosphatase]) indicating that RWA-SA2 partially overcomes the resistance activated by the *Dn* gene. 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 moderate resistance of Tugela Dn in response to RWA-SA2.

Proteins/enzymes that were identified that are unique to this study which have not been previously reported to be implicated in the RWA-wheat interaction are, Photosystem II D2 protein, Photosystem II protein D1, Dehydrin COR410, S-adenosylmethionine synthase, 2-Cys peroxiredoxin BAS1, Chlorophyll a-b binding protein and Protein-L-isoaspartate O-methyltransferase.

Based on the proteomic data which is further supported by the photochemical data, we propose a model to depict the main components involved in RWA-SA2 response to Tugela and Tugela Dn. In this model it is hypothesized that RWA probes the plant and once it has established a feeding site, it releases its phytotoxic compounds and binds to receptor proteins embedded in the cell membrane. The Tugela Dn line appears to have a more successful resistance mechanism against the RWA-SA2 biotype. Upon recognition of the aphid in the resistant Tugela Dn line, this activates a cascade of events downstream resulting in the onset of defense response. The photosynthetic machinery of the plant appears to be less unharmed compared to the decline reached in the susceptible cultivar. It would appear that the susceptible plants displays no recognition and therefore has no time to activate the suitable machinery for cell maintenance, leading to energy production loss and eventually cell death. These are a result of chlorophyll breakdown and severe compromised photosynthetic activity. It is proposed that in an incompatible interaction the plant deliberately decrease its photosynthetic capacity and other assimilatory metabolism processes in order to initiate respiration and other processes needed for defense (Scharte *et al*., 2005). The purpose of doing this might seem like suicide but it could be a desperate attempt on survival. However, the susceptible does not appear to show any form of regeneration, thus, surrendering to aphid attack. It has been previously reported that there are no specific proteins that convert a signal about an increase in the intracellular ROS levels to a biochemical response in the cells. However, herein we report that it would appear that the resistant cultivar, Tugela Dn, scavenges ROS by up-regulating 2-Cys peroxiredoxin for use in the hypersensitive response. ROS have regulatory properties in stress conditions and is produced at various sources including NADPH-oxidase receiving electrons through ferredoxin. It still remains questionable which particular ROS plays a signalling role in the chloroplast and how different signalling pathways respond to an increase in the level of different types of ROS.



Indicates what is known for RWA-SA1 on wheat (Adapted from Botha *et al.*, 2005)

Indicates what is proposed for RWA-SA2 on wheat

Future Work

The findings from this study suggest that the 2DE method employed was suitable for the identification of proteins exclusively expressed as part of Tugela Dn's resistance mechanism. However, in order to improve reproducibility and gel resolution in future studies, IPG strips of narrow pH ranges should be used to improve resolution of spots in the second dimension especially putting more emphasis on protein spots in the acidic region. Broad range IPG strips provide one with a general impression of the distribution of proteins with regards to their pI values but the use of narrow pH range strips will allow for improved spot resolution (Gorg, 1991). Another area that would also assist with improved protein spot resolution is to increase the IPG strip as well as the size of the second dimension gel from 11cm to a 17cm gel. A further aspect of the work that must be addressed on the involvement of specific proteins in the resistance mechanism is to use the following complementary techniques:

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 at different time intervals. These will then serve as potential protein markers to develop resistant wheat strains to counteract the aphid attack on wheat plants.

qPCR

The up-regulation of transcription of specific key gene regulating the pathways identified in this study will be monitored using qPCR. An induction of any of these genes would be a strategy to overcome pest attack to keep the photosystem I stable and energy production going and a reduction might be to force energy flow in a different direction

Adding elicitors

A resistant and susceptible wheat cultivar will be taken and treated with various elicitors such as JA, SA or ABA and resistance to RWA will be scored. This will give an idea if the pathway was common to both. If the protein was only expressed in RWA infested plants, it should become RWA resistant. The deposition of callose will also be evaluated using fluorescent microscopy.

Measuring particular enzyme activity

Enzymes within the unique pathways identified in this study will be selected and the enzymatic activity will be monitored in resistant cultivars to RWA at different time frames. For example, Fructose-1,6-bisphosphatase catalyzes the reaction converting D-fructose 1,6-bisphosphate to D-fructose 6-phosphate in carbohydrate biosynthesis. It would be interesting to see whether the induction of FBP would overcome the step inhibited by thioredoxin.

Measuring role of Ca2+ in the response mechanism

Confocal microscopy will be used to monitor Ca2+ fluxes in resistant and susceptible cultivars at various time frames, in response to RWA infestation. The role of Ca2+ signalling proteins as a potential defense strategy of Aphid-Resistant Wheat plants have also been recently studied. It would be interesting to see whether virulent Diuraphis noxia Aphids causes over-expression of calcium signaling proteins and may give useful insights into this relationship.

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 quantitated by liquid chromatography mass spectrometry (LC/MS).

Phytoalexin production

The presence or absence of phytoalexins will be monitored at different timeframes to confirm what role they play in the resistance mechanism.

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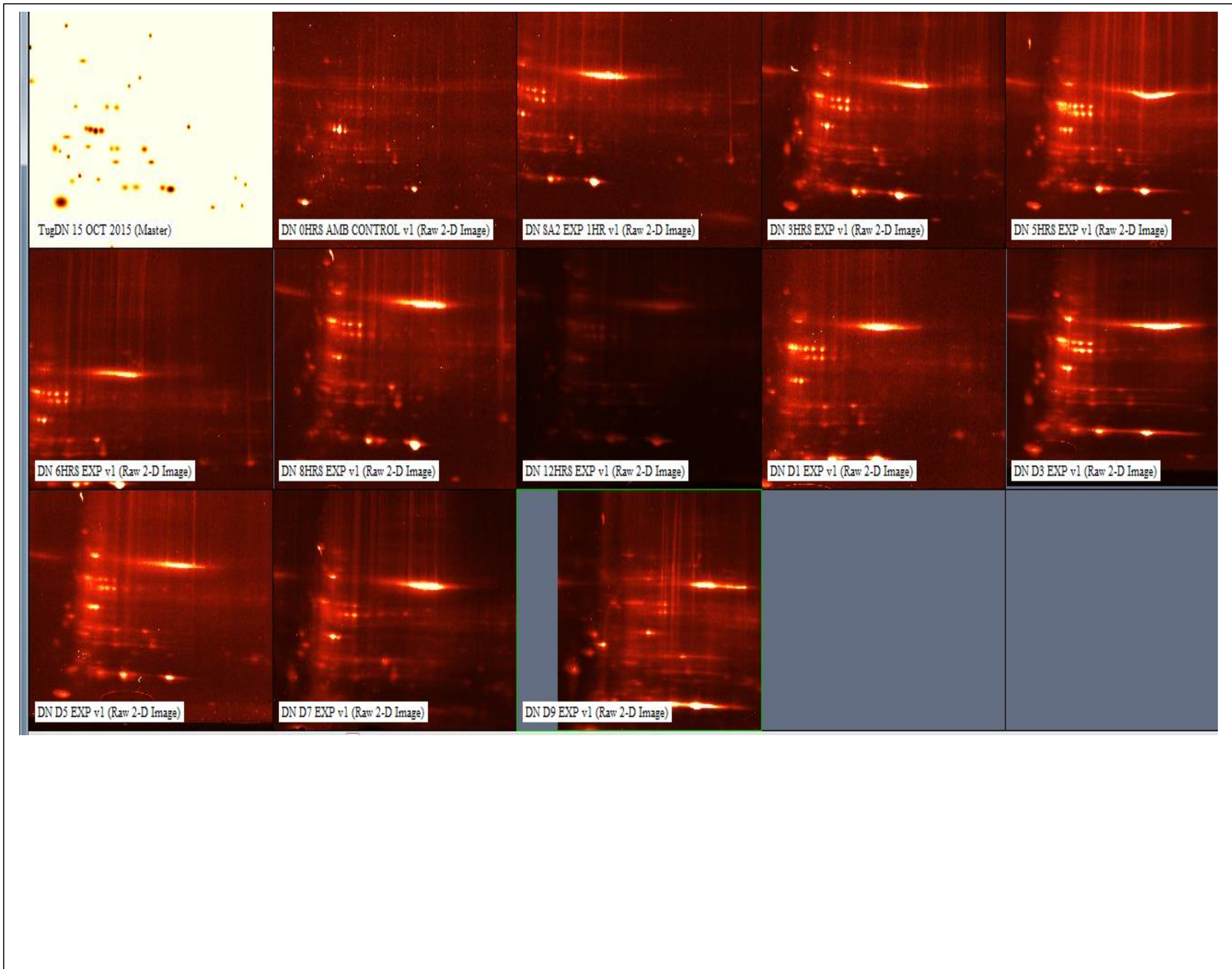
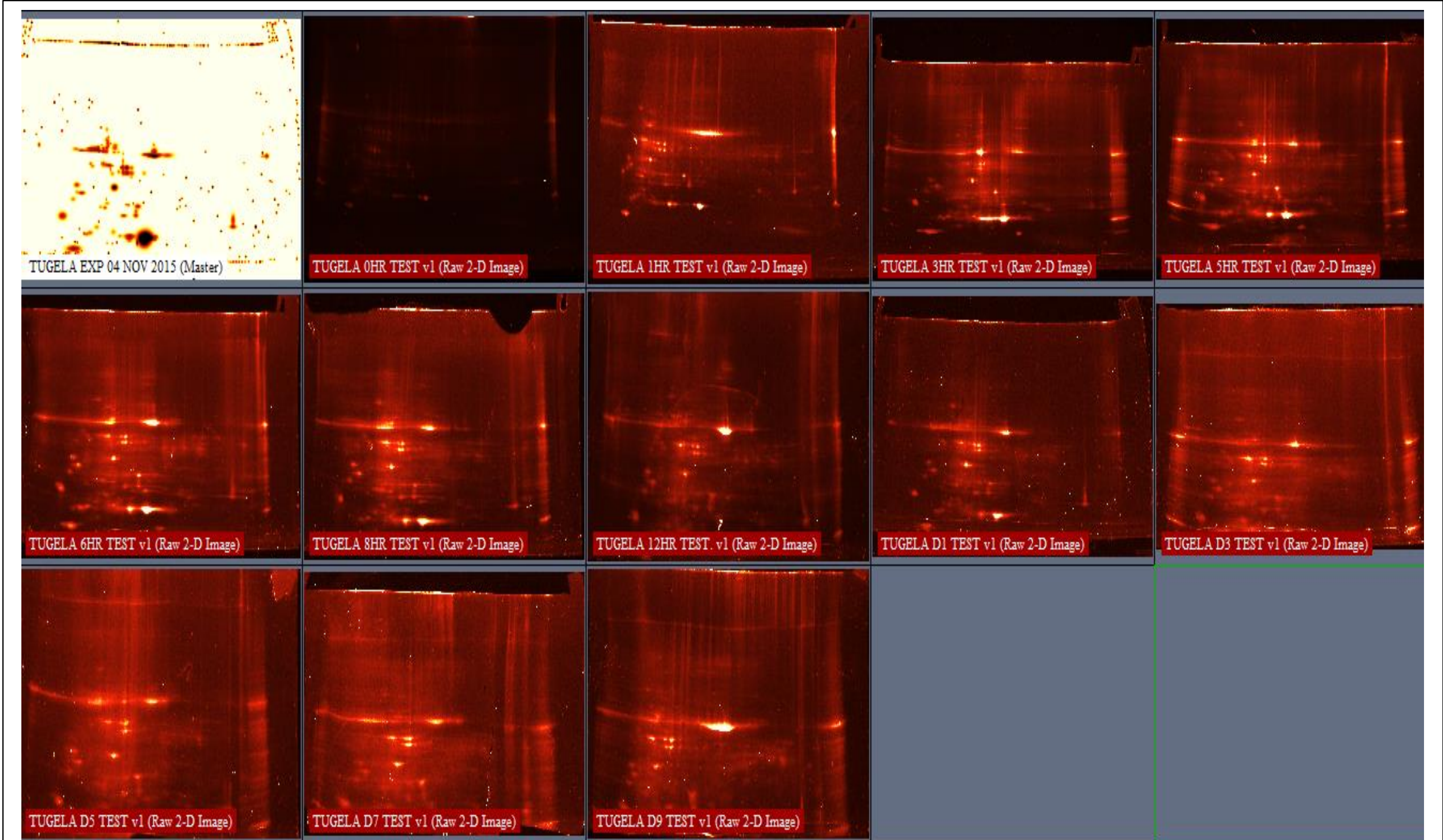
Appendix 1:
Results for Chapter 4

OUTPUT RESULTS FOR TUGELA-*DN*

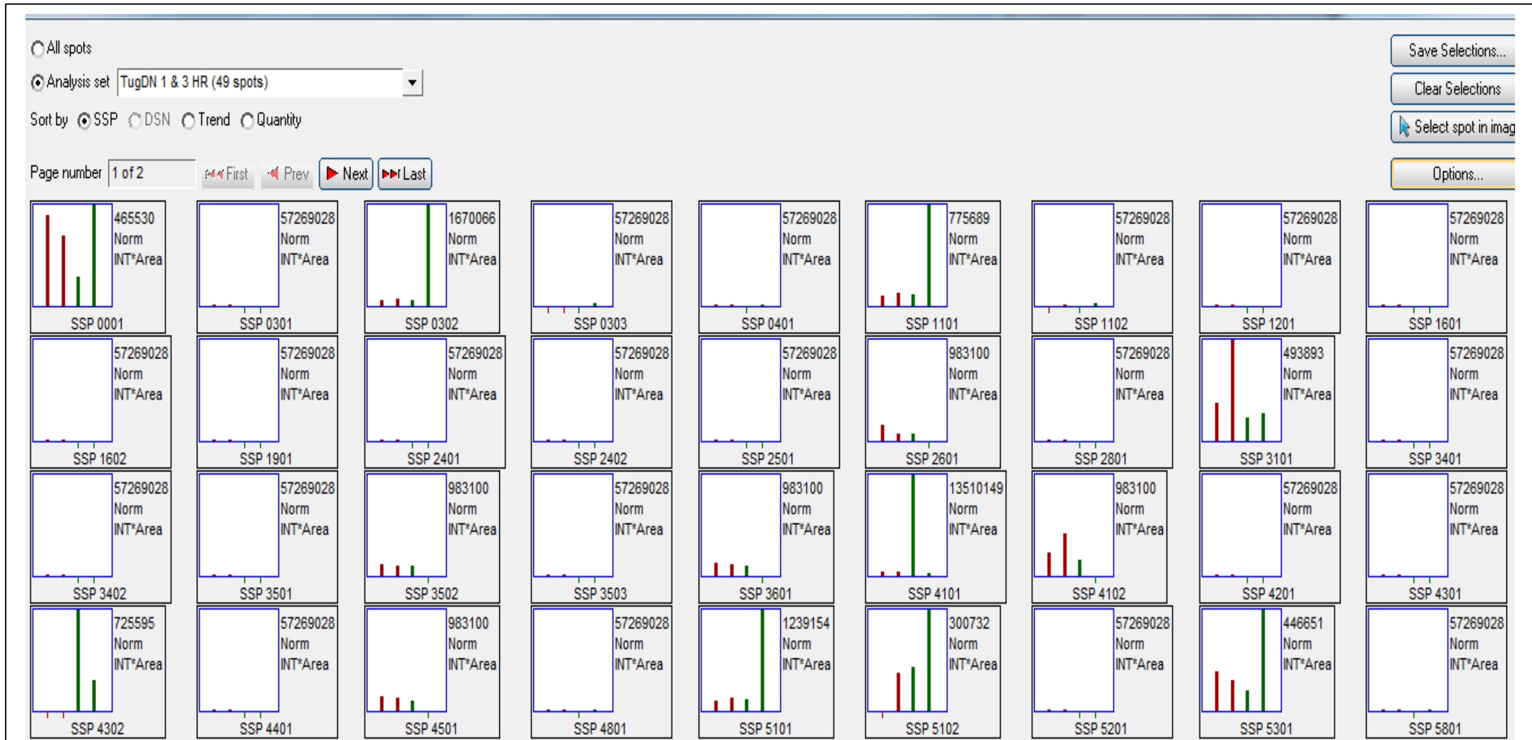


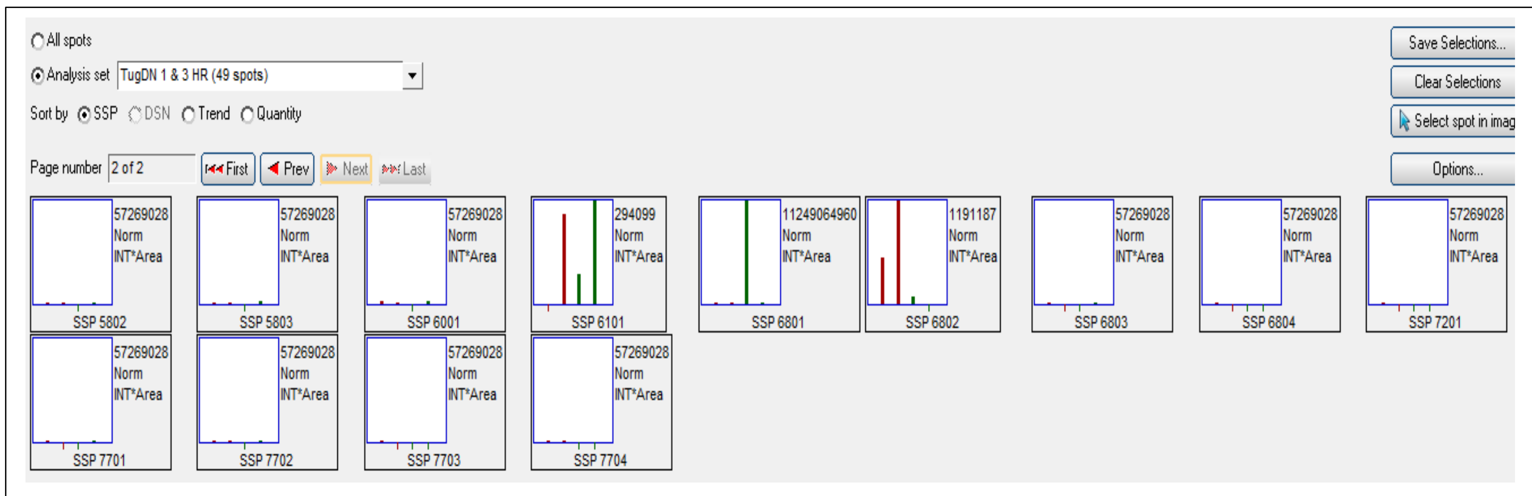
OUTPUT RESULTS FOR TUGELA

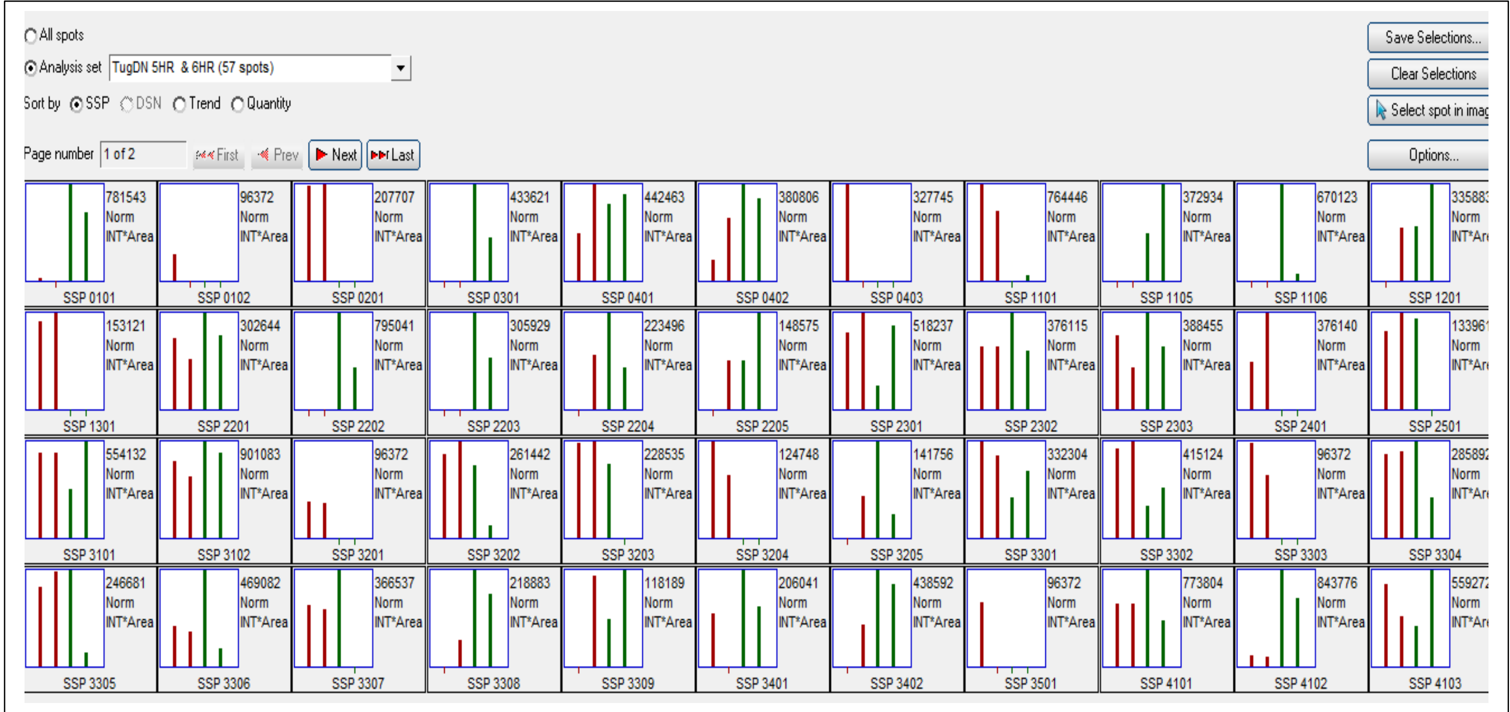


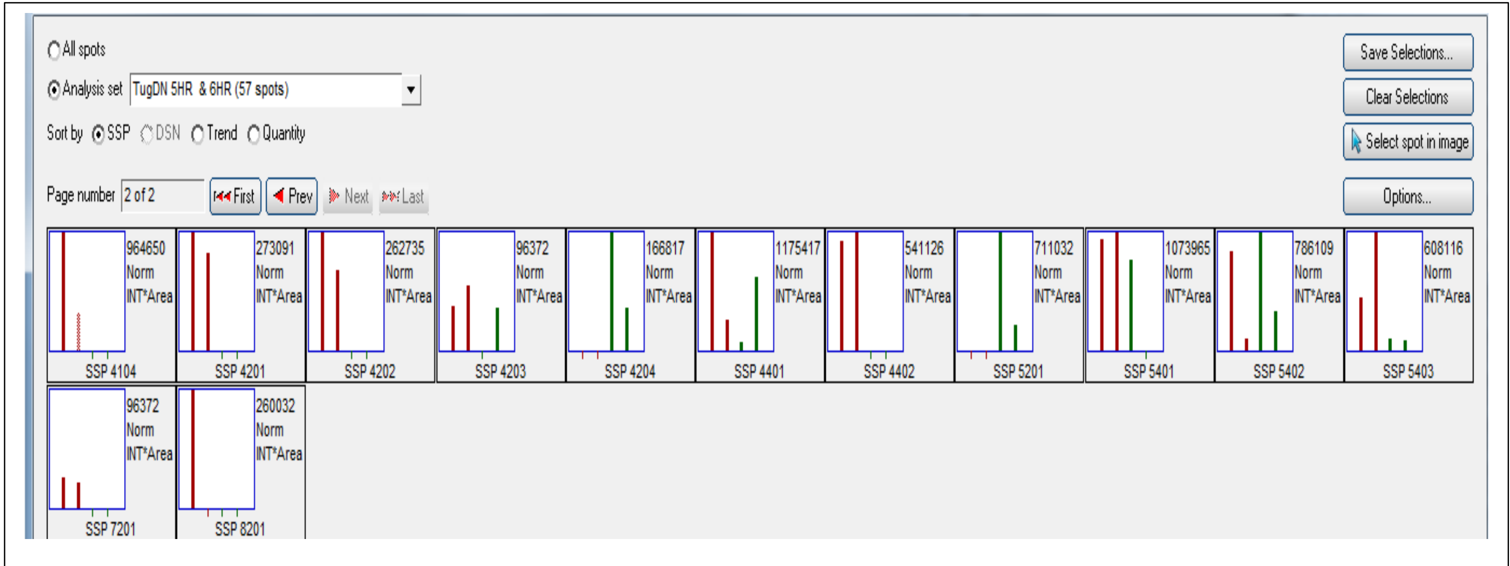
QUANTITY GRAPH REPORTS

<u>TUGELA-*DN*</u>






All spots
Analysis set TugDN 5HR & 6HR (57 spots)
Sort by SSP DSN Trend Quantity
Page number 1 of 2
First Prev Next Last
Save Selections...
Clear Selections
Options...
781543 Norm INT*Area SSP 0101
96372 Norm INT*Area SSP 0102
207707 Norm INT*Area SSP 0201
433621 Norm INT*Area SSP 0301
442463 Norm INT*Area SSP 0401
380806 Norm INT*Area SSP 0402
327745 Norm INT*Area SSP 0403
764446 Norm INT*Area SSP 1101
372934 Norm INT*Area SSP 1105
670123 Norm INT*Area SSP 1106
Norm SSP 1201
153121 Norm INT*Area SSP 1301
302644 Norm INT*Area SSP 2201
795041 Norm INT*Area SSP 2202
305929 Norm INT*Area SSP 2203
223496 Norm INT*Area SSP 2204
148575 Norm INT*Area SSP 2205
518237 Norm INT*Area SSP 2301
376115 Norm INT*Area SSP 2302
388455 Norm INT*Area SSP 2303
376140 Norm INT*Area SSP 2401
Norm SSP 2501
554132 Norm INT*Area SSP 3101
901083 Norm INT*Area SSP 3102
96372 Norm INT*Area SSP 3201
261442 Norm INT*Area SSP 3202
228535 Norm INT*Area SSP 3203
124748 Norm INT*Area SSP 3204
141756 Norm INT*Area SSP 3205
332304 Norm INT*Area SSP 3301
415124 Norm INT*Area SSP 3302
96372 Norm INT*Area SSP 3303
Norm SSP 3304
246681 Norm INT*Area SSP 3305
469082 Norm INT*Area SSP 3306
366537 Norm INT*Area SSP 3307
218883 Norm INT*Area SSP 3308
118189 Norm INT*Area SSP 3309
206041 Norm INT*Area SSP 3401
438592 Norm INT*Area SSP 3402
96372 Norm INT*Area SSP 3501
773804 Norm INT*Area SSP 4101
843776 Norm INT*Area SSP 4102
Norm SSP 4103


All spots
Analysis set TugDN 5HR & 6HR (57 spots)
Sort by SSP DSN Trend Quantity
Page number 2 of 2
First Prev Next Last
Save Selections...
Clear Selections
Select spot in image
Options...
964650 Norm INT*Area SSP 4104
273091 Norm INT*Area SSP 4201
262735 Norm INT*Area SSP 4202
96372 Norm INT*Area SSP 4203
166817 Norm INT*Area SSP 4204
1175417 Norm INT*Area SSP 4401
541126 Norm INT*Area SSP 4402
711032 Norm INT*Area SSP 5201
1073965 Norm INT*Area SSP 5401
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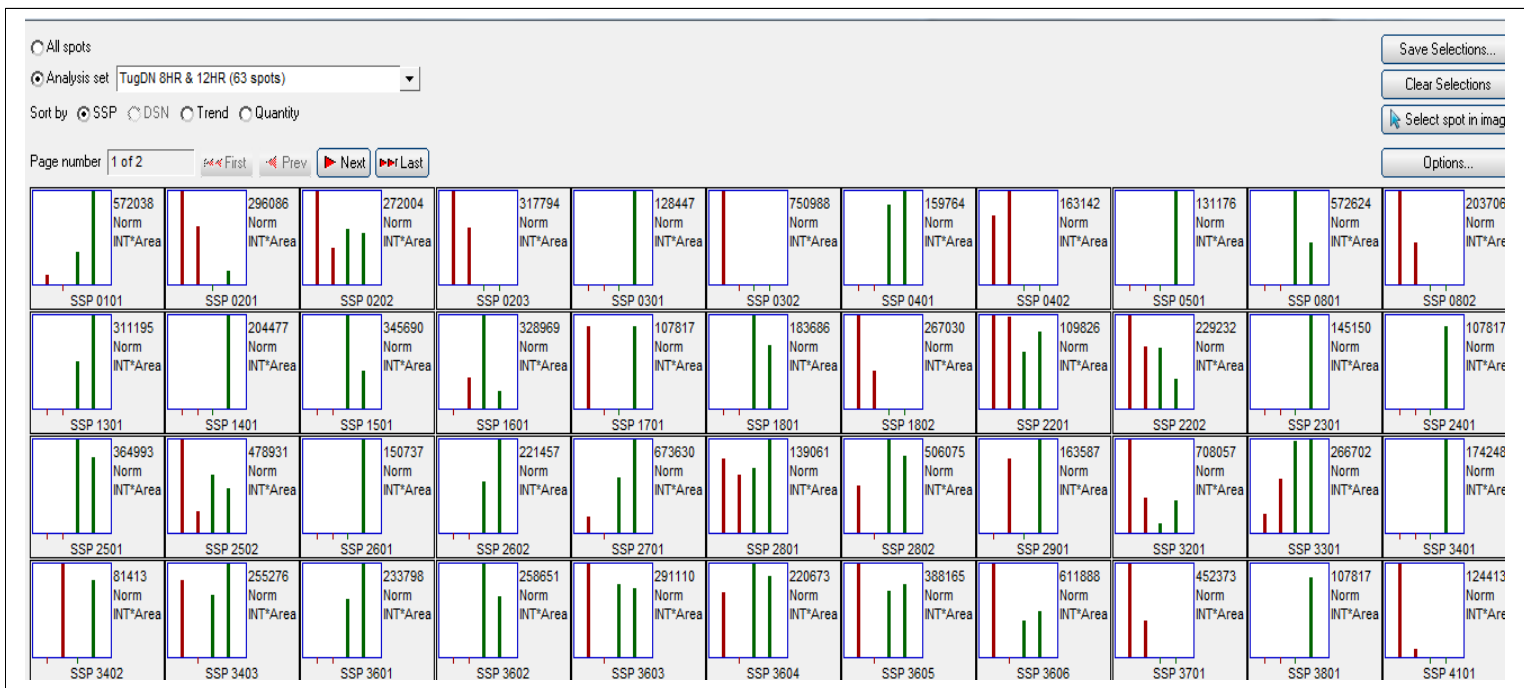
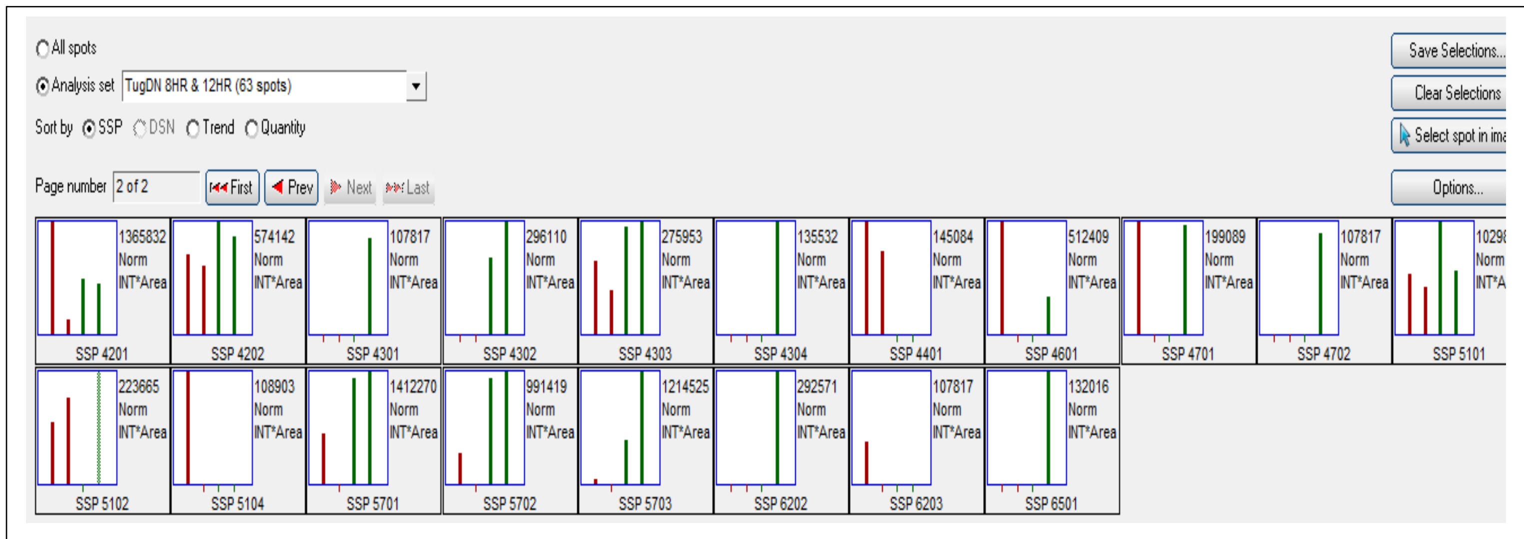
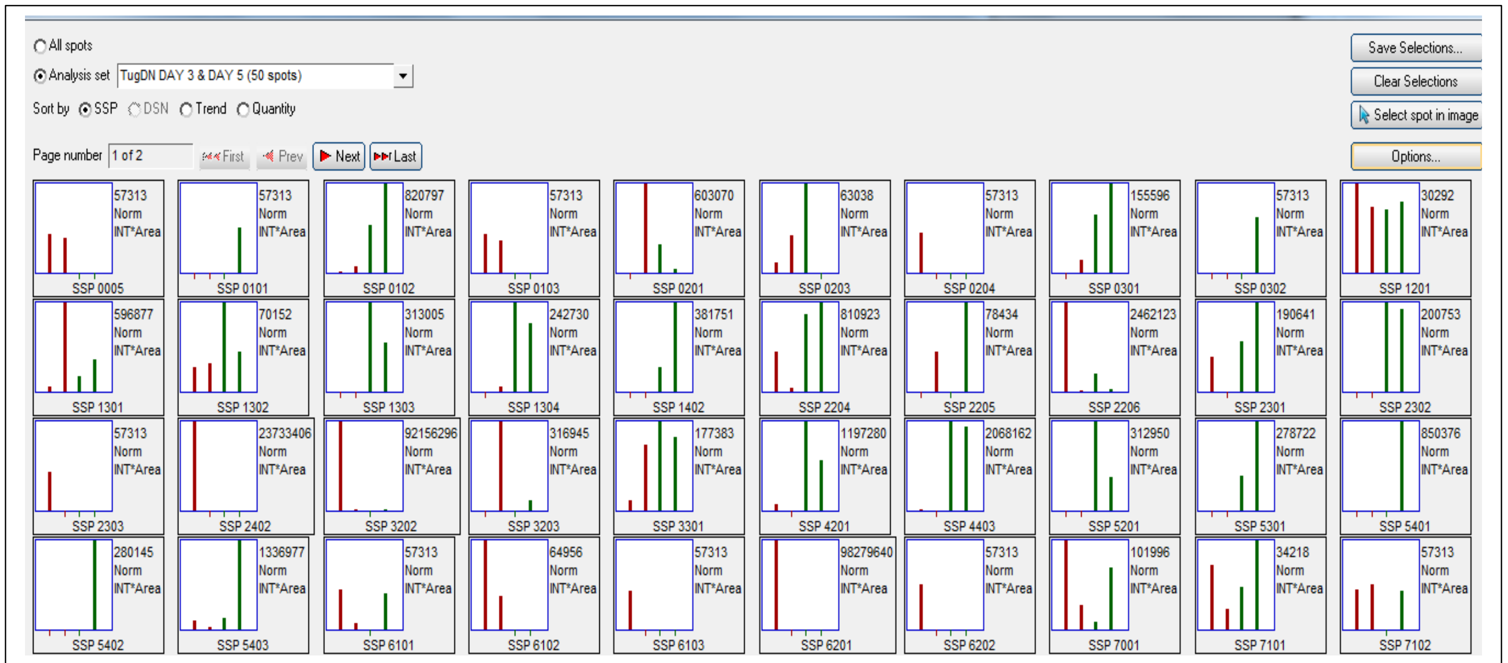
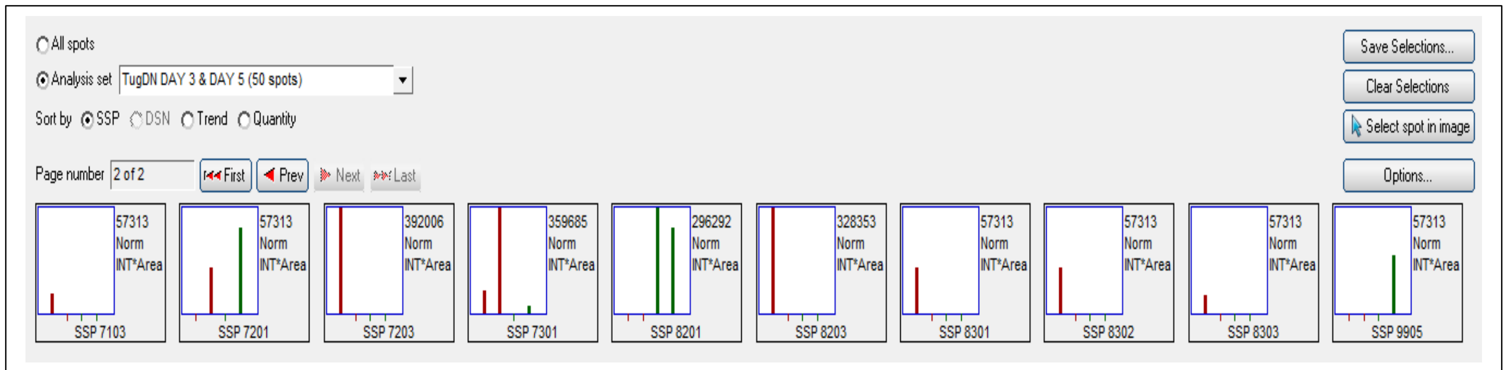

All spots
Analysis set TugDN 8HR & 12HR (63 spots)
Sort by SSP DSN Trend Quantity
Page number 1 of 2
First Prev Next Last
Save Selections...
Clear Selections
Select spot in imag
Options...
572038 Norm INT*Area
SSP 0101
296086 Norm INT*Area
SSP 0201
272004 Norm INT*Area
SSP 0202
317794 Norm INT*Area
SSP 0203
128447 Norm INT*Area
SSP 0301
750988 Norm INT*Area
SSP 0302
159764 Norm INT*Area
SSP 0401
163142 Norm INT*Area
SSP 0402
131176 Norm INT*Area
SSP 0501
572624 Norm INT*Area
SSP 0801
203706 Norm INT*Are
SSP 0802
311195 Norm INT*Area
SSP 1301
204477 Norm INT*Area
SSP 1401
345690 Norm INT*Area
SSP 1501
328969 Norm INT*Area
SSP 1601
107817 Norm INT*Area
SSP 1701
183686 Norm INT*Area
SSP 1801
267030 Norm INT*Area
SSP 1802
109826 Norm INT*Area
SSP 2201
229232 Norm INT*Area
SSP 2202
145150 Norm INT*Area
SSP 2301
107817 Norm INT*Are
SSP 2401
364993 Norm INT*Area
SSP 2501
478931 Norm INT*Area
SSP 2502
150737 Norm INT*Area
SSP 2601
221457 Norm INT*Area
SSP 2602
673630 Norm INT*Area
SSP 2701
139061 Norm INT*Area
SSP 2801
506075 Norm INT*Area
SSP 2802
163587 Norm INT*Area
SSP 2901
708057 Norm INT*Area
SSP 3201
266702 Norm INT*Area
SSP 3301
174248 Norm INT*Are
SSP 3401
81413 Norm INT*Area
SSP 3402
255276 Norm INT*Area
SSP 3403
233798 Norm INT*Area
SSP 3601
258651 Norm INT*Area
SSP 3602
291110 Norm INT*Area
SSP 3603
220673 Norm INT*Area
SSP 3604
388165 Norm INT*Area
SSP 3605
611888 Norm INT*Area
SSP 3606
452373 Norm INT*Area
SSP 3701
107817 Norm INT*Area
SSP 3801
124413 Norm INT*Are
SSP 4101


All spots
Analysis set TugDN 8HR & 12HR (63 spots)
Sort by SSP DSN Trend Quantity
Page number 2 of 2
First Prev Next Last
Save Selections...
Clear Selections
Select spot in ima
Options...
1365832 Norm INT*Area
SSP 4201
574142 Norm INT*Area
SSP 4202
107817 Norm INT*Area
SSP 4301
296110 Norm INT*Area
SSP 4302
275953 Norm INT*Area
SSP 4303
135532 Norm INT*Area
SSP 4304
145084 Norm INT*Area
SSP 4401
512409 Norm INT*Area
SSP 4601
199089 Norm INT*Area
SSP 4701
107817 Norm INT*Area
SSP 4702
Norm INT*A
SSP 5101
223665 Norm INT*Area
SSP 5102
108903 Norm INT*Area
SSP 5104
1412270 Norm INT*Area
SSP 5701
991419 Norm INT*Area
SSP 5702
1214525 Norm INT*Area
SSP 5703
292571 Norm INT*Area
SSP 6202
107817 Norm INT*Area
SSP 6203
132016 Norm INT*Area
SSP 6501


All spots
Analysis set TugDN DAY 3 & DAY 5 (50 spots)
Sort by SSP DSN Trend Quantity
Page number 1 of 2
First Prev Next Last
Save Selections...
Clear Selections
Select spot in image
Options...
57313 Norm INT*Area SSP 0005
57313 Norm INT*Area SSP 0101
820797 Norm INT*Area SSP 0102
57313 Norm INT*Area SSP 0103
603070 Norm INT*Area SSP 0201
63038 Norm INT*Area SSP 0203
57313 Norm INT*Area SSP 0204
155596 Norm INT*Area SSP 0301
57313 Norm INT*Area SSP 0302
30292 Norm INT*Area SSP 1201
596877 Norm INT*Area SSP 1301
70152 Norm INT*Area SSP 1302
313005 Norm INT*Area SSP 1303
242730 Norm INT*Area SSP 1304
381751 Norm INT*Area SSP 1402
810923 Norm INT*Area SSP 2204
78434 Norm INT*Area SSP 2205
2462123 Norm INT*Area SSP 2206
190641 Norm INT*Area SSP 2301
200753 Norm INT*Area SSP 2302
57313 Norm INT*Area SSP 2303
23733406 Norm INT*Area SSP 2402
92156296 Norm INT*Area SSP 3202
316945 Norm INT*Area SSP 3203
177383 Norm INT*Area SSP 3301
1197280 Norm INT*Area SSP 4201
2068162 Norm INT*Area SSP 4403
312950 Norm INT*Area SSP 5201
278722 Norm INT*Area SSP 5301
850376 Norm INT*Area SSP 5401
280145 Norm INT*Area SSP 5402
1336977 Norm INT*Area SSP 5403
57313 Norm INT*Area SSP 6101
64956 Norm INT*Area SSP 6102
57313 Norm INT*Area SSP 6103
98279640 Norm INT*Area SSP 6201
57313 Norm INT*Area SSP 6202
101996 Norm INT*Area SSP 7001
34218 Norm INT*Area SSP 7101
57313 Norm INT*Area SSP 7102


All spots
Analysis set TugDN DAY 3 & DAY 5 (50 spots)
Sort by SSP DSN Trend Quantity
Page number 2 of 2
First Prev Next Last
Save Selections...
Clear Selections
Select spot in image
Options...
57313 Norm INT*Area SSP 7103
57313 Norm INT*Area SSP 7201
392006 Norm INT*Area SSP 7203
359685 Norm INT*Area SSP 7301
296292 Norm INT*Area SSP 8201
328353 Norm INT*Area SSP 8203
57313 Norm INT*Area SSP 8301
57313 Norm INT*Area SSP 8302
57313 Norm INT*Area SSP 8303
57313 Norm INT*Area SSP 9905

All spots
Analysis set TugDN D3, D7 &D9 (41 spots)
Sort by SSP DSN Trend Quantity
Page number 1 of 2
First Prev Next Last
Save Selections...
Clear Selections
Select spot in image
Options...
90337 Norm INT*Area SSP 0201
90337 Norm INT*Area SSP 1201
90337 Norm INT*Area SSP 1202
75024 Norm INT*Area SSP 1203
114441 Norm INT*Area SSP 1204
90337 Norm INT*Area SSP 1205
90337 Norm INT*Area SSP 1206
291350 Norm INT*Area SSP 1207
102201 Norm INT*Area SSP 1208
775518 Norm INT*Area SSP 1209
90337 Norm INT*Area SSP 1210
90337 Norm INT*Area SSP 1211
4628725 Norm INT*Area SSP 1212
90337 Norm INT*Area SSP 1213
90337 Norm INT*Area SSP 1214
90337 Norm INT*Area SSP 1215
143708 Norm INT*Area SSP 1216
117646 Norm INT*Area SSP 1217
350089 Norm INT*Area SSP 1218
90337 Norm INT*Area SSP 1219
142477 Norm INT*Area SSP 1220
371736 Norm INT*Area SSP 1301
90337 Norm INT*Area SSP 2201
371736 Norm INT*Area SSP 2202
90337 Norm INT*Area SSP 2203
90337 Norm INT*Area SSP 2204
90337 Norm INT*Area SSP 2205
95737 Norm INT*Area SSP 2206
69737 Norm INT*Area SSP 2207
143505 Norm INT*Area SSP 2208
90337 Norm INT*Area SSP 2209
96602 Norm INT*Area SSP 2210
338079 Norm INT*Area SSP 2211
183681 Norm INT*Area SSP 2212
312246 Norm INT*Area SSP 2214
371736 Norm INT*Area SSP 2302

All spots

Analysis set TugDN D3, D7 &D9 (41 spots)

Sort by SSP DSN Trend Quantity

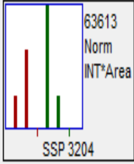
Page number 2 of 2 First Prev Next Last

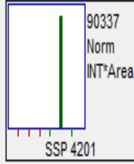
Save Selections...

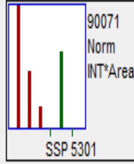
Clear Selections

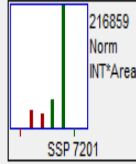
Select spot in image

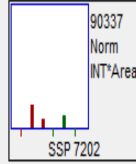
Options...











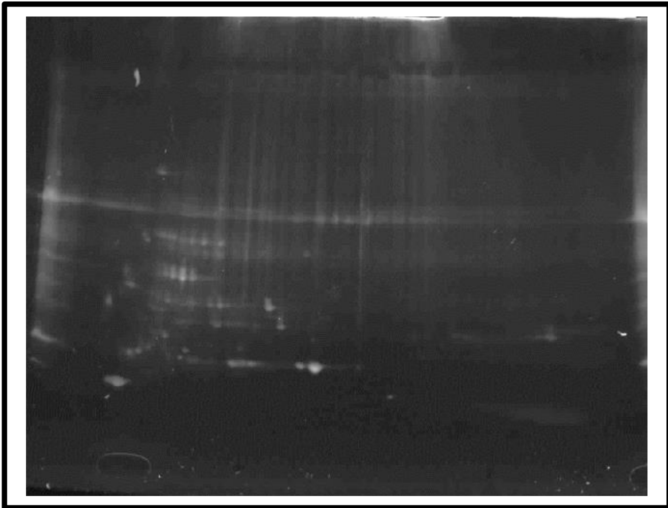
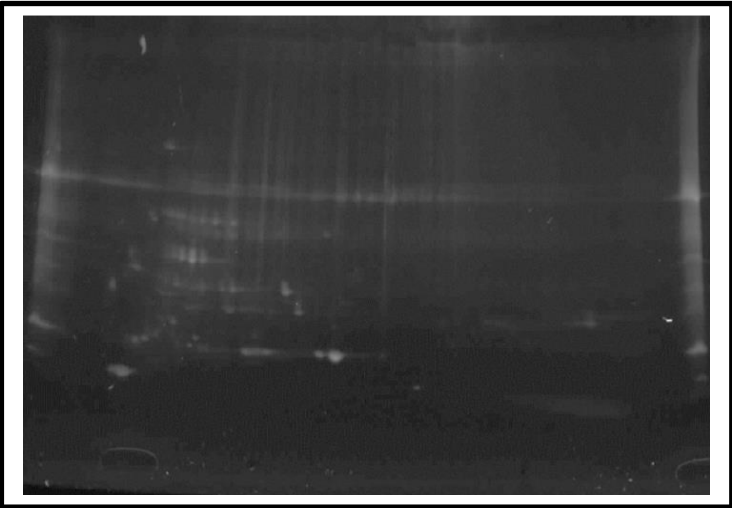
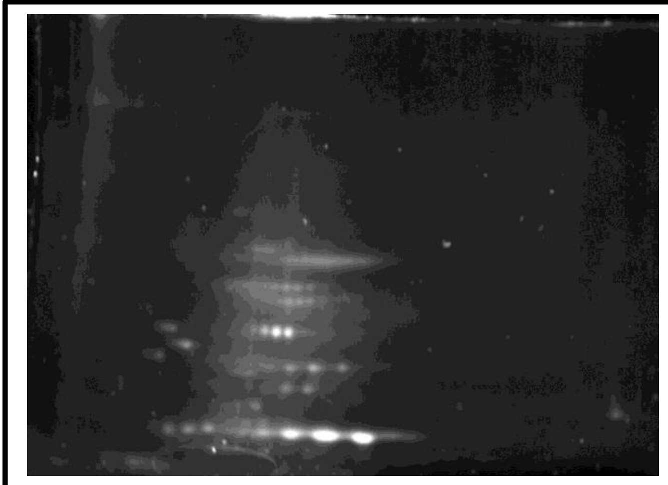
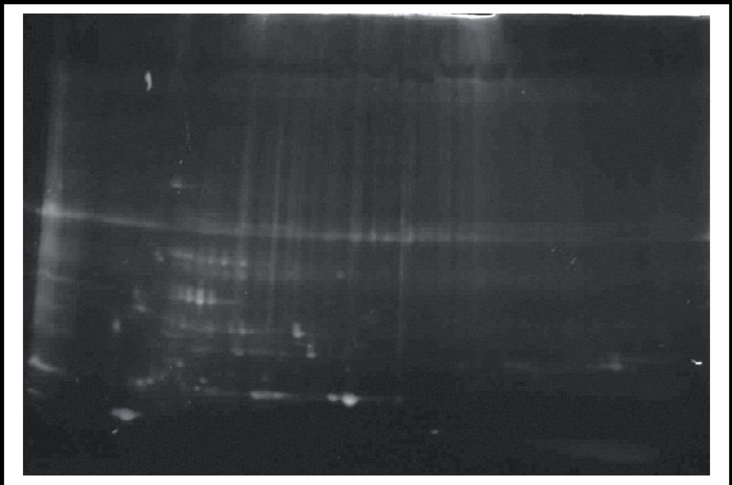
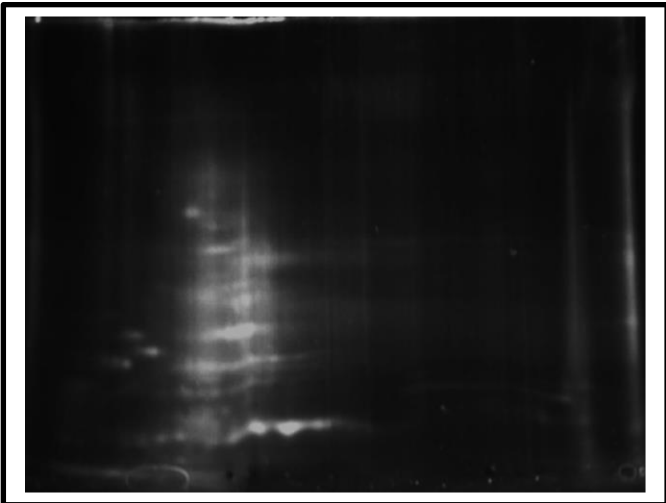
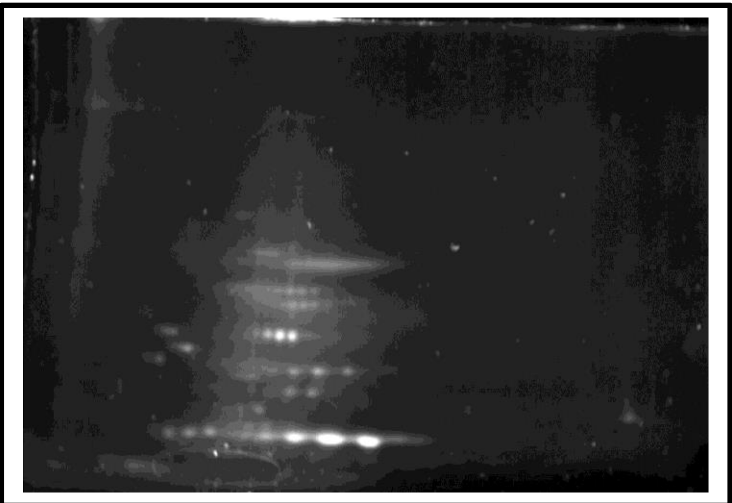
TUGELA

All spots
Analysis set TUG TEST 0HR AND1HR (42 spots)
Sort by SSP DSN Trend Quantity
Page number 1 of 1 First Prev Next Last
Save Selections...
Clear Selections
Select spot in imag
Options...

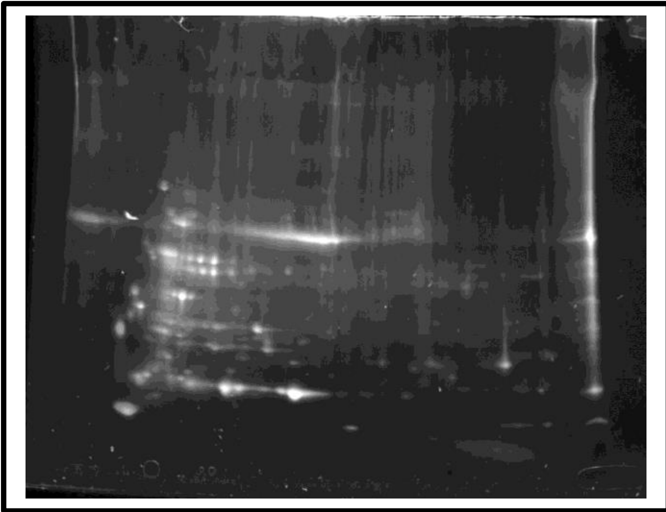
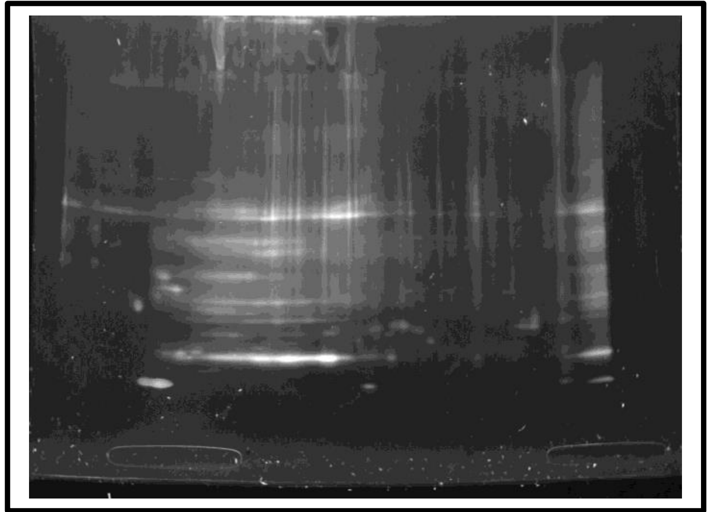
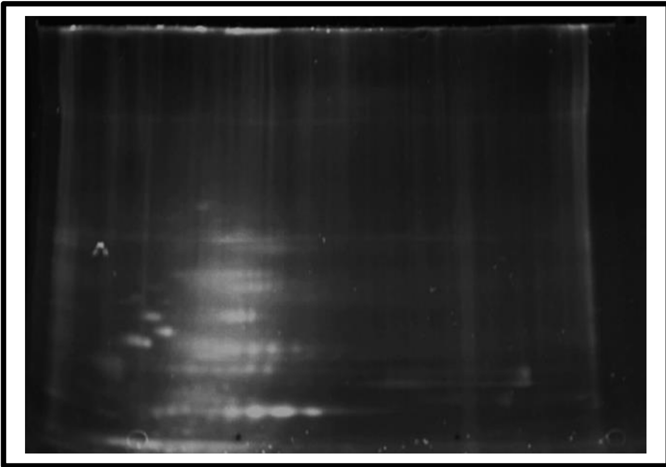
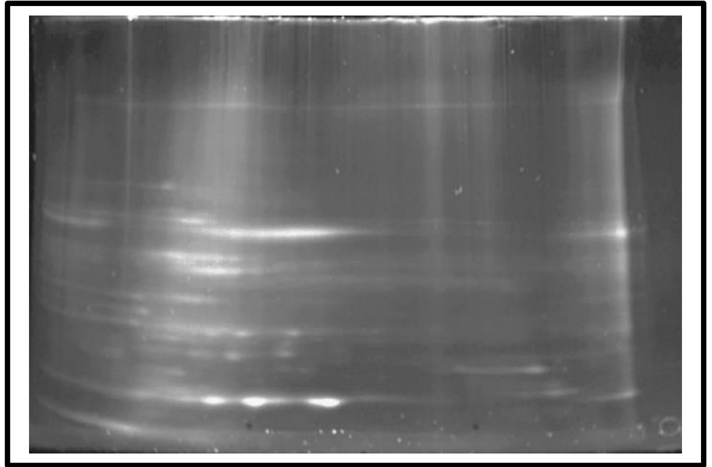
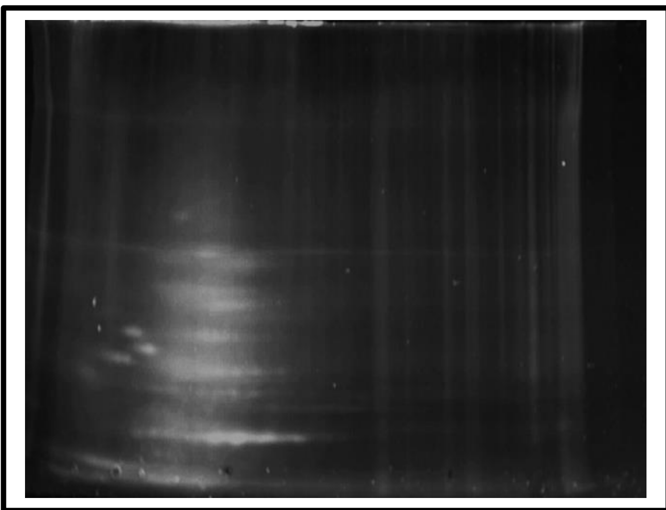
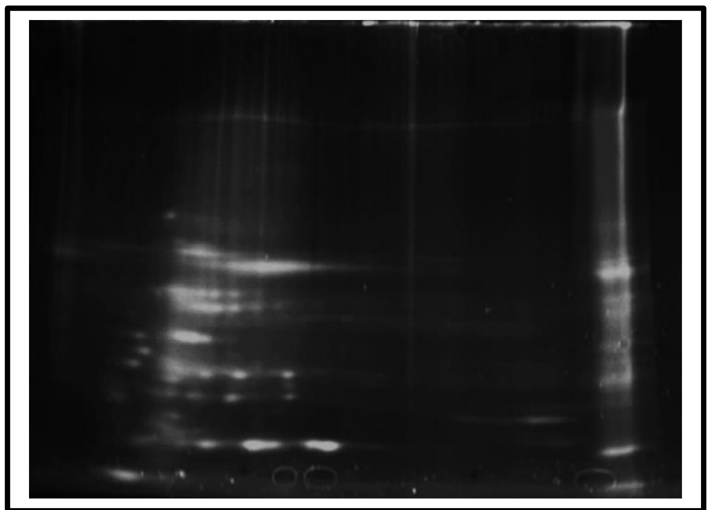
482717 Norm INT*Area SSP 1201
105685 Norm INT*Area SSP 1501
156571 Norm INT*Area SSP 1802
55312 Norm INT*Area SSP 1803
71479 Norm INT*Area SSP 2301
51153 Norm INT*Area SSP 2302
70386 Norm INT*Area SSP 2501
47485 Norm INT*Area SSP 3401
77435 Norm INT*Area SSP 3601
357999 Norm INT*Area SSP 3702
84992 Norm INT*Ar SSP 4301
39964 Norm INT*Area SSP 4302
74020 Norm INT*Area SSP 4501
38339 Norm INT*Area SSP 4502
82021 Norm INT*Area SSP 4503
140981 Norm INT*Area SSP 4601
316829 Norm INT*Area SSP 4602
80310 Norm INT*Area SSP 4701
38339 Norm INT*Area SSP 5401
74732 Norm INT*Area SSP 5402
71594 Norm INT*Area SSP 5403
58314 Norm INT*Ar SSP 5501
111854 Norm INT*Area SSP 5502
121409 Norm INT*Area SSP 5601
77655 Norm INT*Area SSP 5701
96738 Norm INT*Area SSP 5702
92177 Norm INT*Area SSP 5703
414713 Norm INT*Area SSP 6201
114412 Norm INT*Area SSP 6202
111883 Norm INT*Area SSP 6203
38339 Norm INT*Area SSP 6401
81552 Norm INT*Area SSP 6402
12599 Norm INT*Ar SSP 6501
164791 Norm INT*Area SSP 7201
1281216 Norm INT*Area SSP 7202
78818 Norm INT*Area SSP 7401
38339 Norm INT*Area SSP 7601
583957 Norm INT*Area SSP 7801
394498 Norm INT*Area SSP 7802
200663 Norm INT*Area SSP 7803
106446 Norm INT*Area SSP 8301
295839 Norm INT*Area SSP 8801

PROTEIN EXPRESSION PROFILES FOR SELECTED TIMEFRAMES

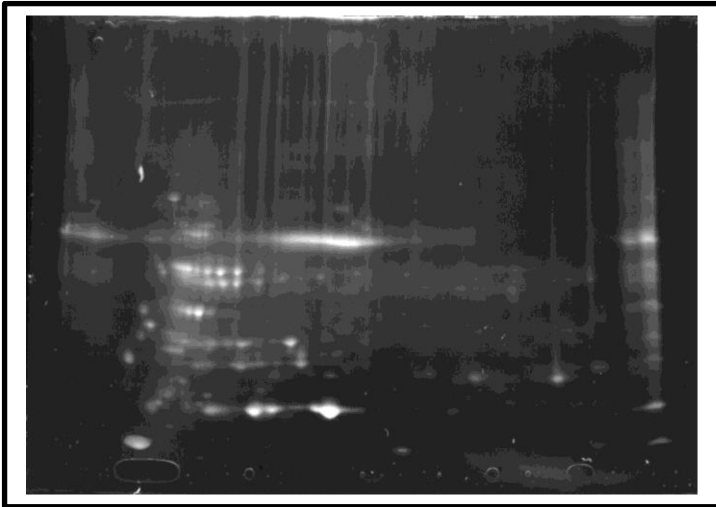
0HR TUGELA*DN* TEST GELS **0HR TUGELA*DN* CONTROL GELS**

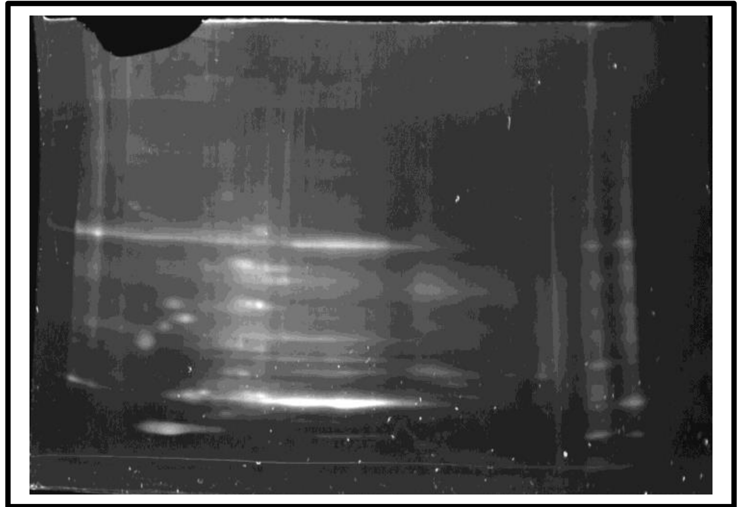







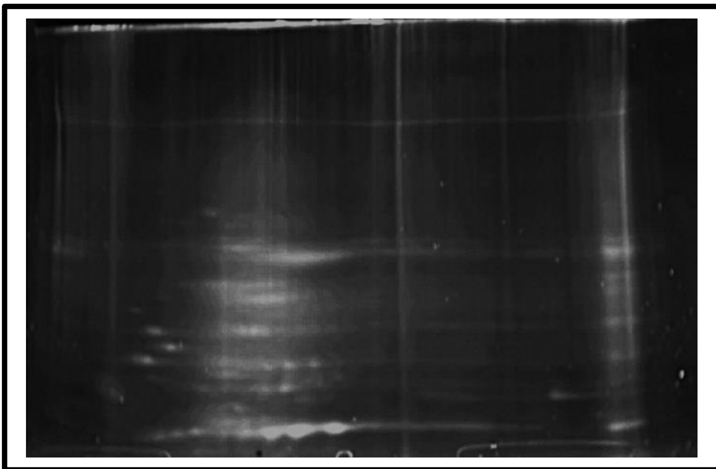
3HR TUGELA*DN* TEST GELS | **3HR TUGELA*DN* CONTROL GELS**

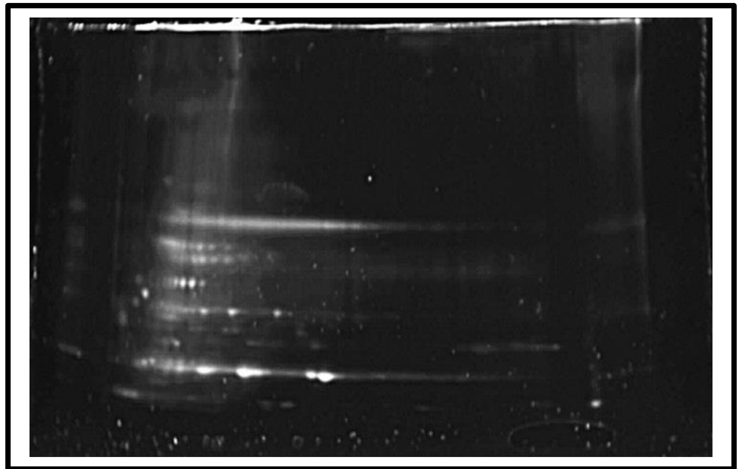







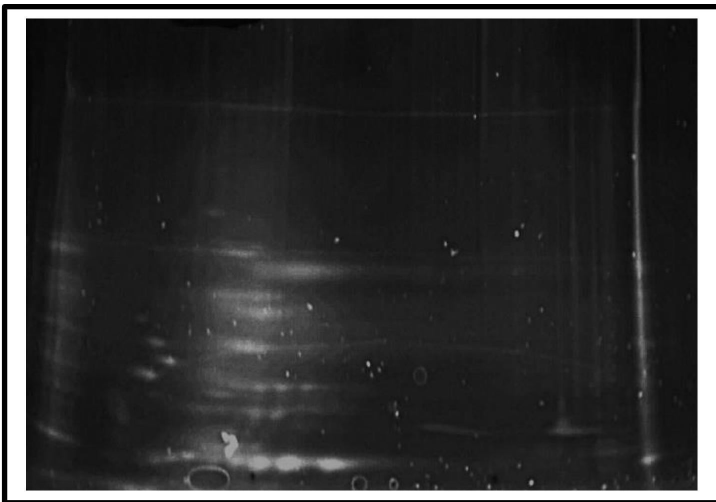
6HR TUGELA*DN* TEST GELS **6HR TUGELA*DN* CONTROL GELS**

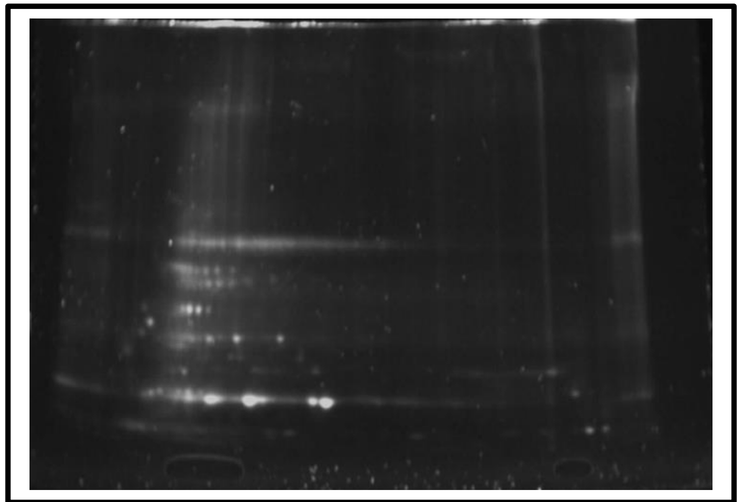


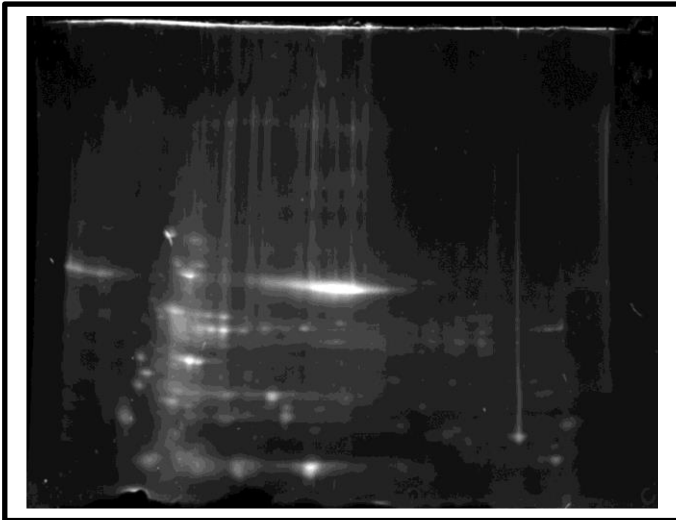
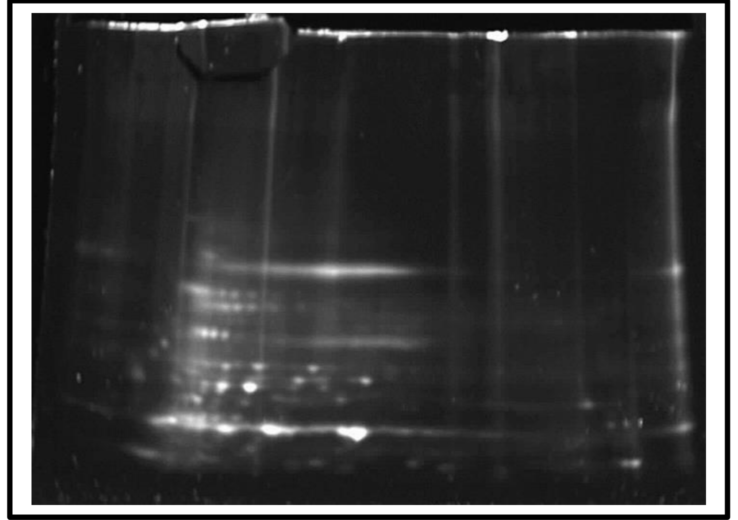
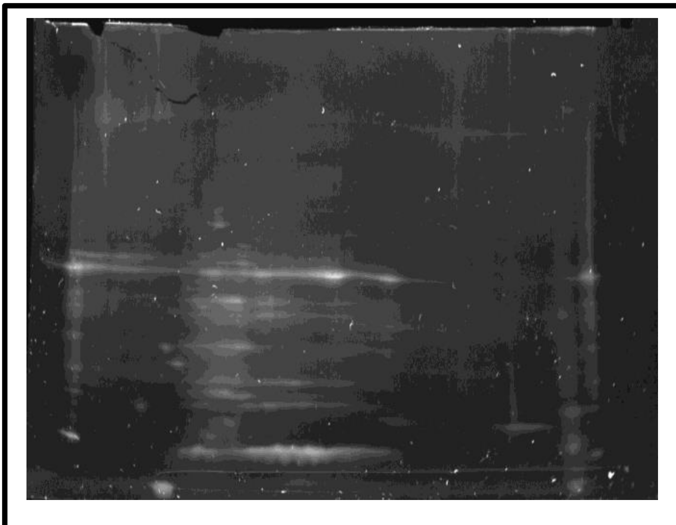
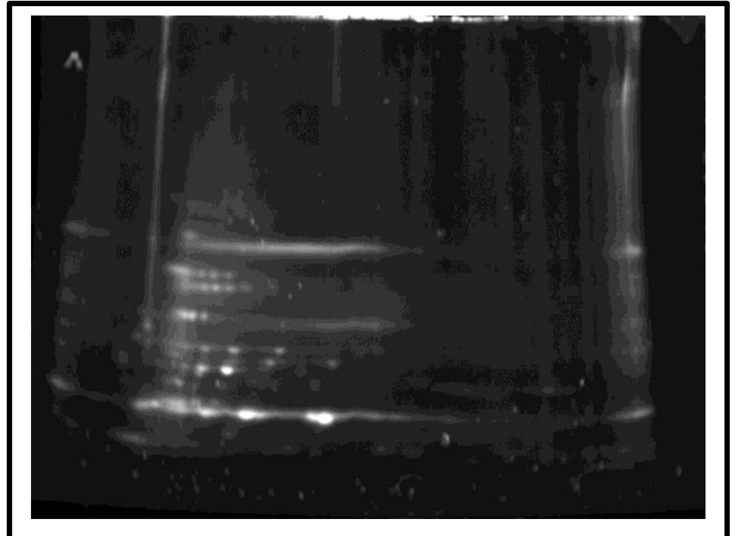
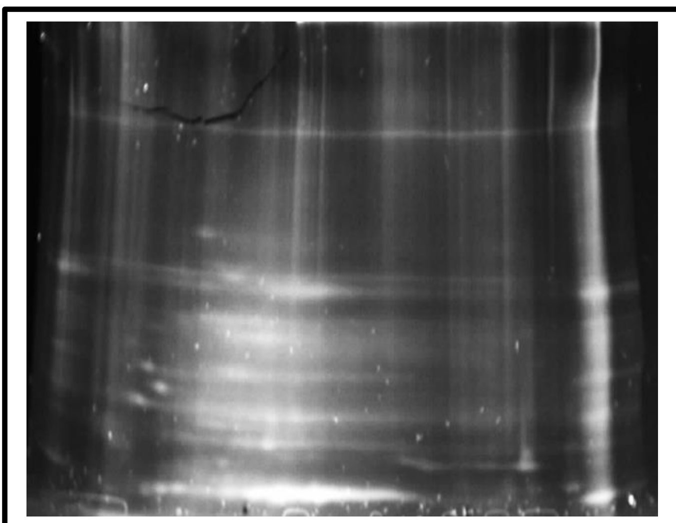
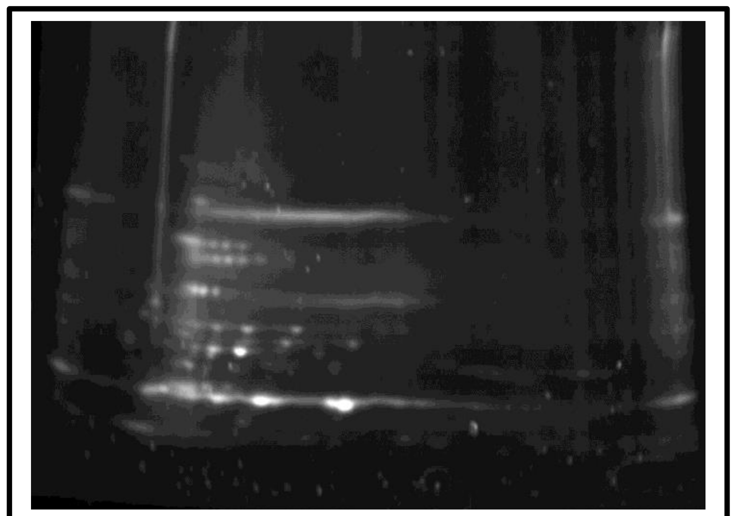




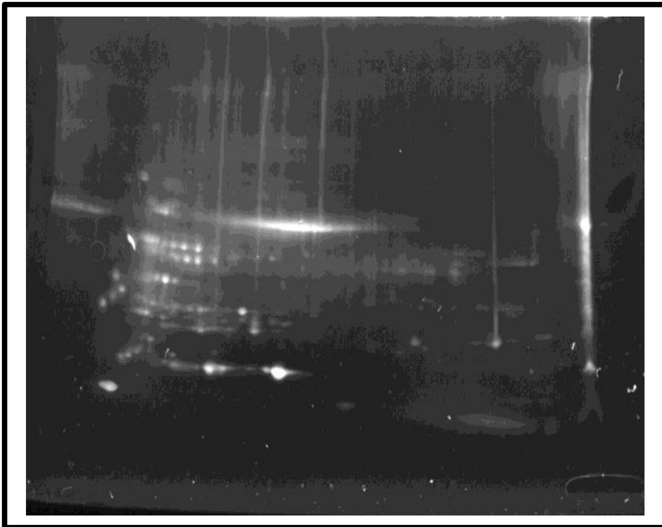


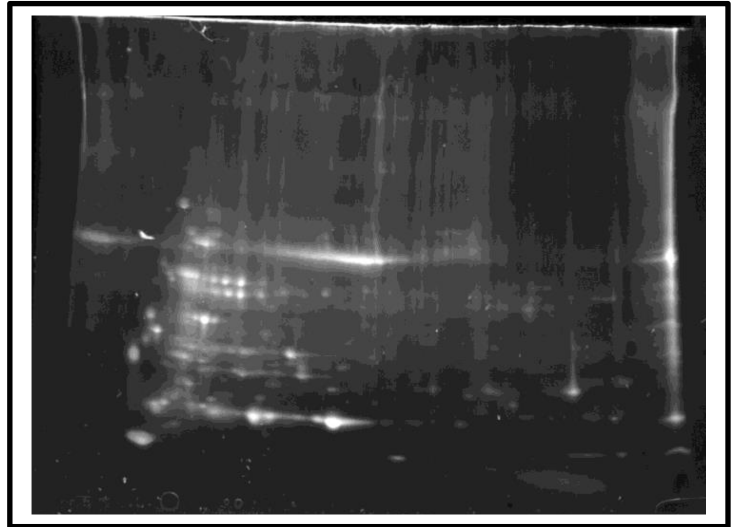


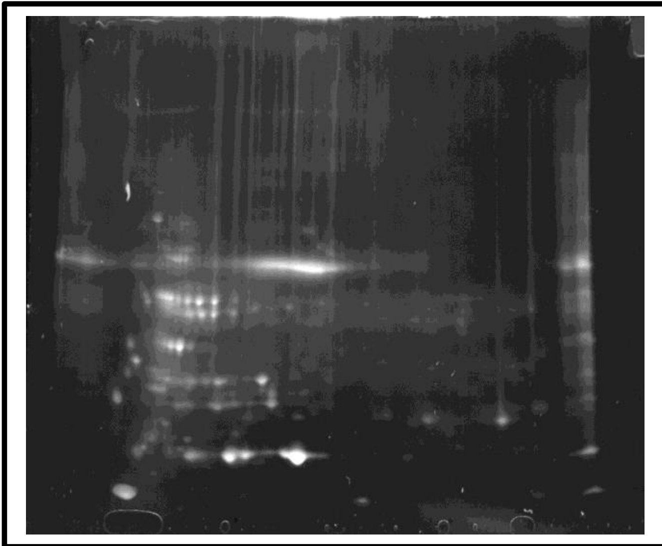


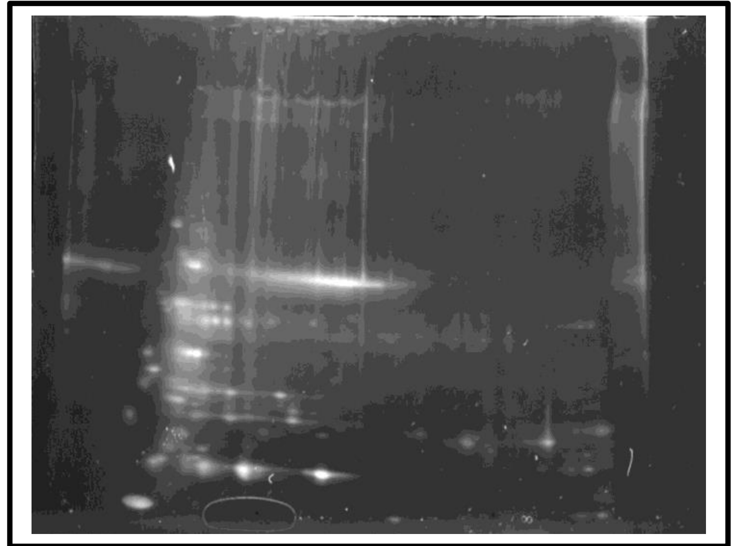
DAY 7 TUGELA*DN* TEST GELS	**DAY 7 TUGELA*DN* CONTROL GELS**
	
	
	

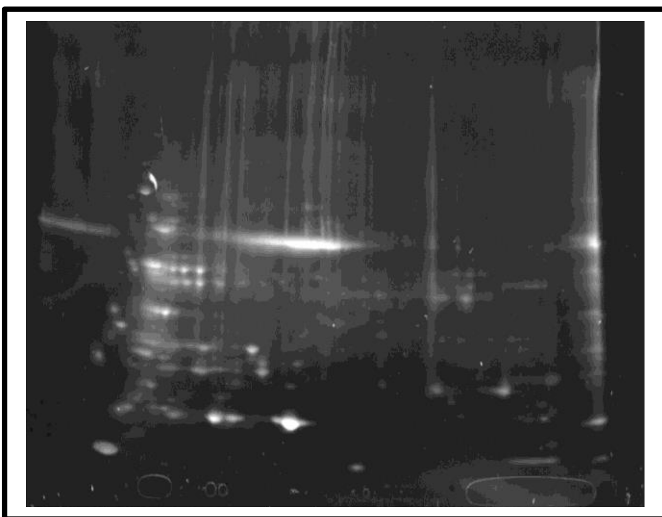
0HR TUGELA SUSCEPTIBLE TEST GELS **0 HR TUGELA SUSCEPTIBLE CONTROLS**

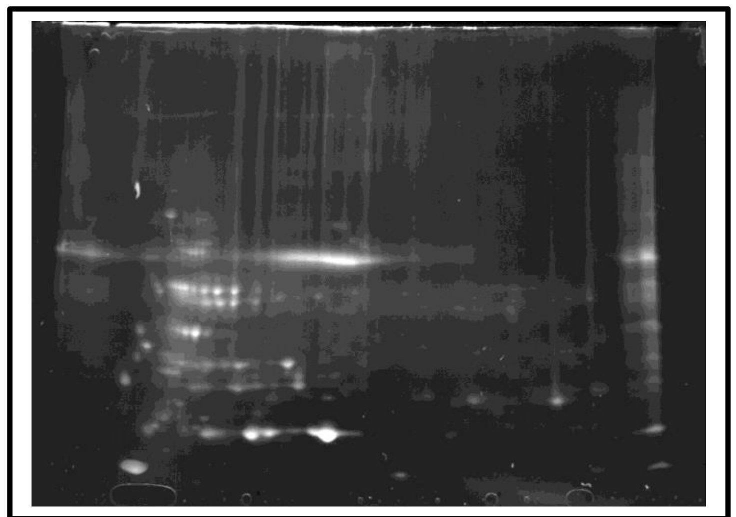




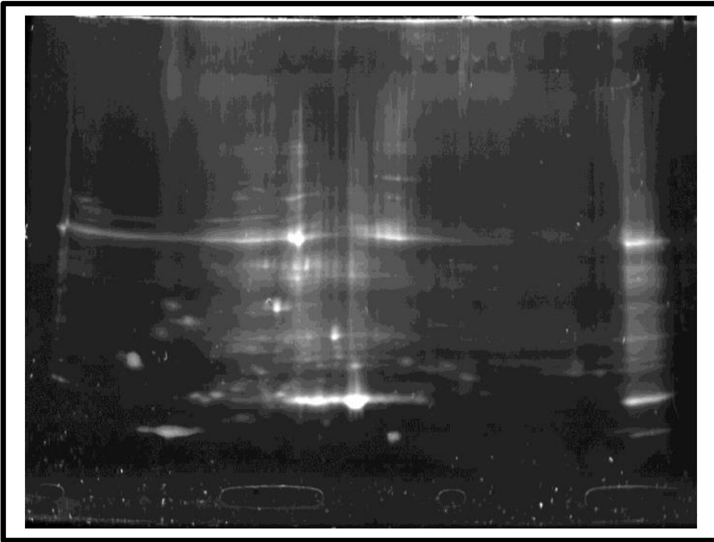


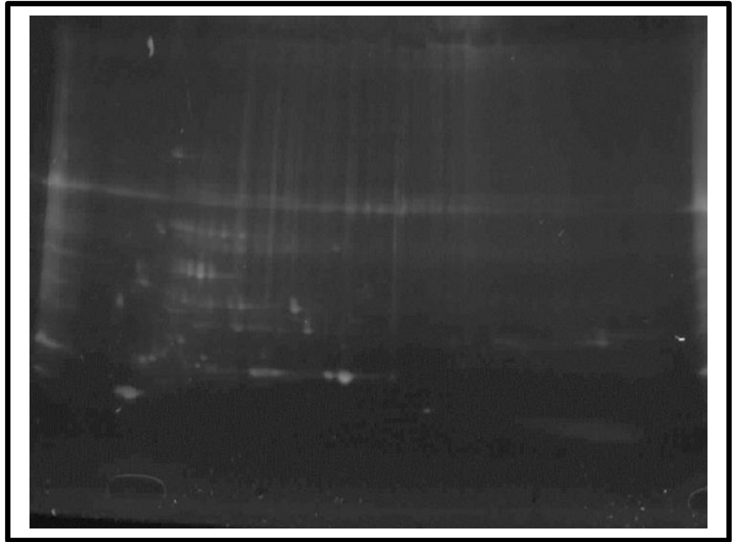


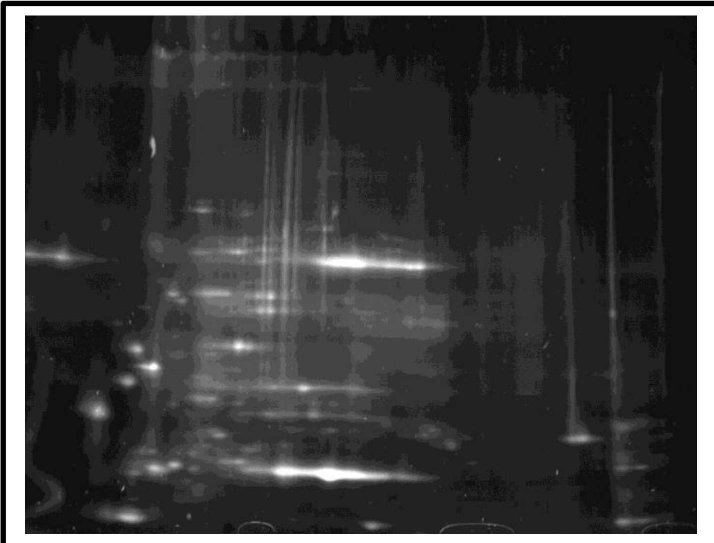


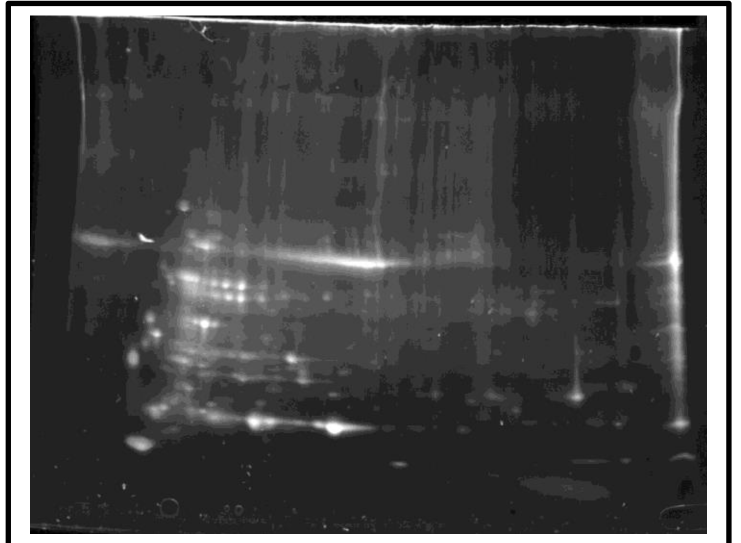


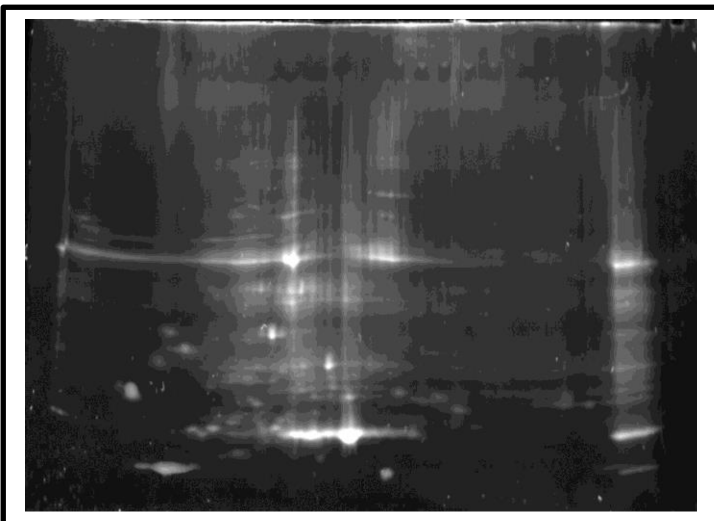
3HR TUGELA SUSCEPTIBLE TEST GELS **3HR TUGELA SUSCEPTIBLE CONTROLS**

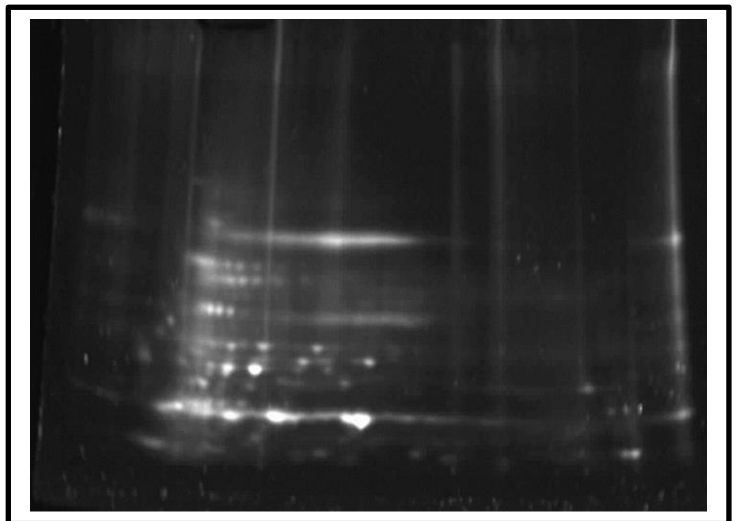






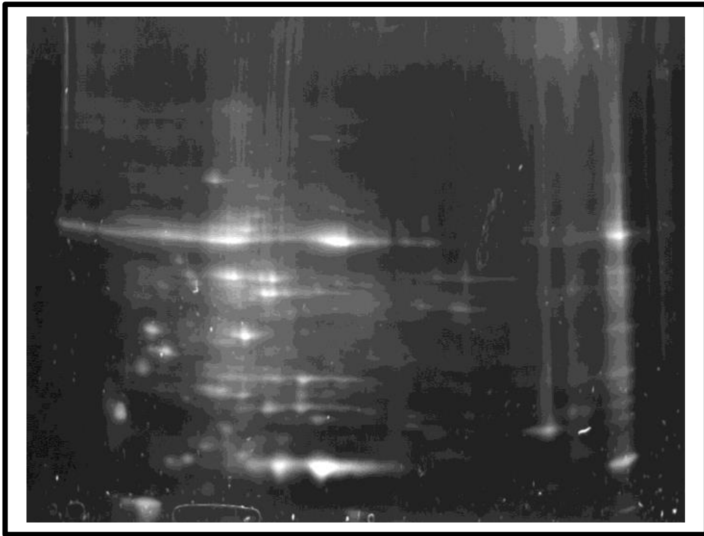


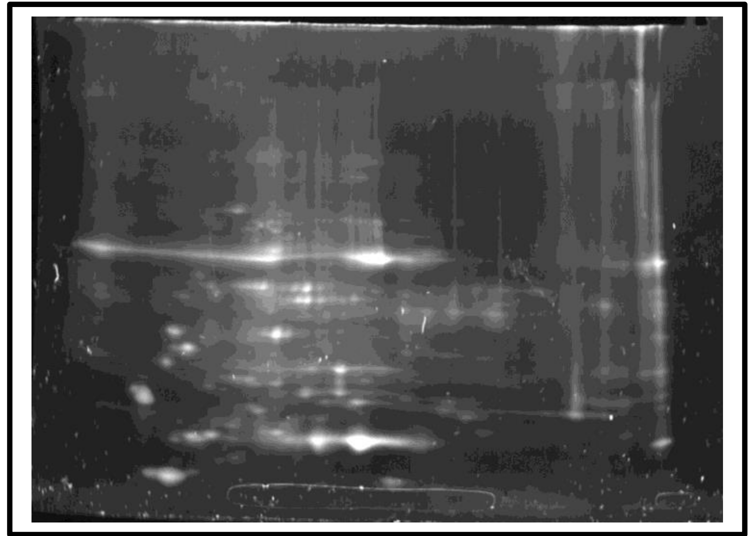


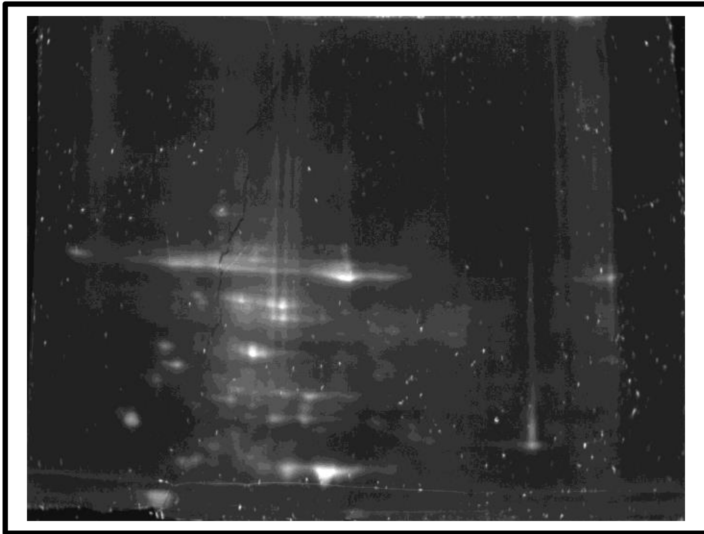


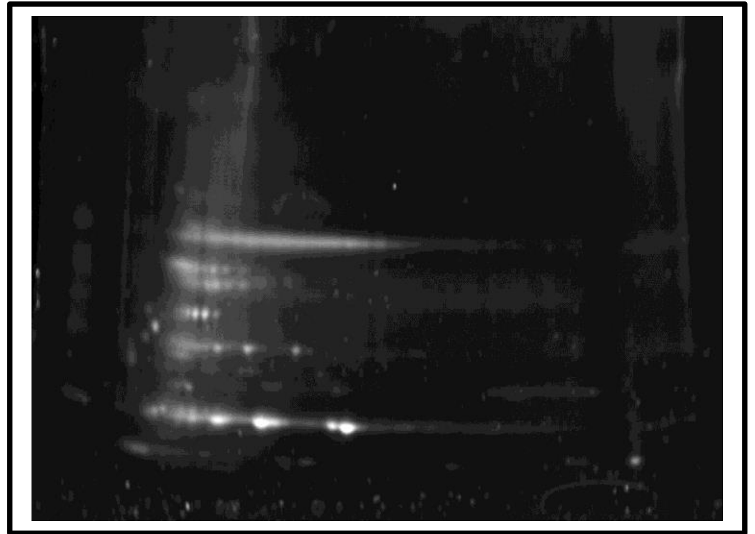
6HR TUGELA SUSCEPTIBLE TEST GELS

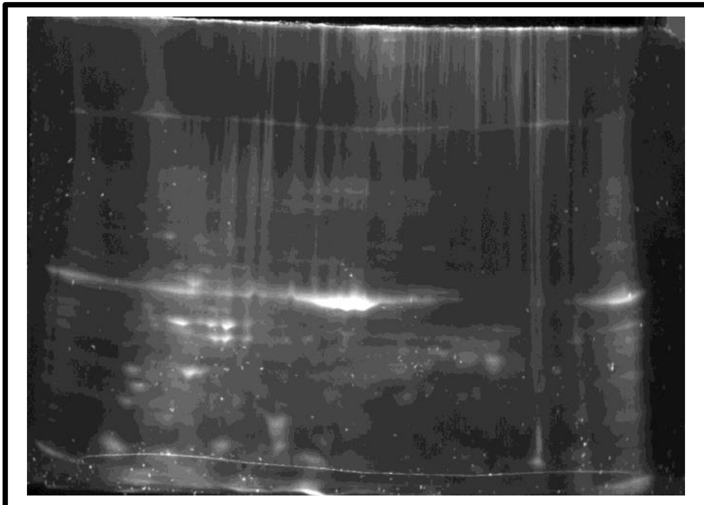
6HR TUGELA SUSCEPTIBLE CONTROLS

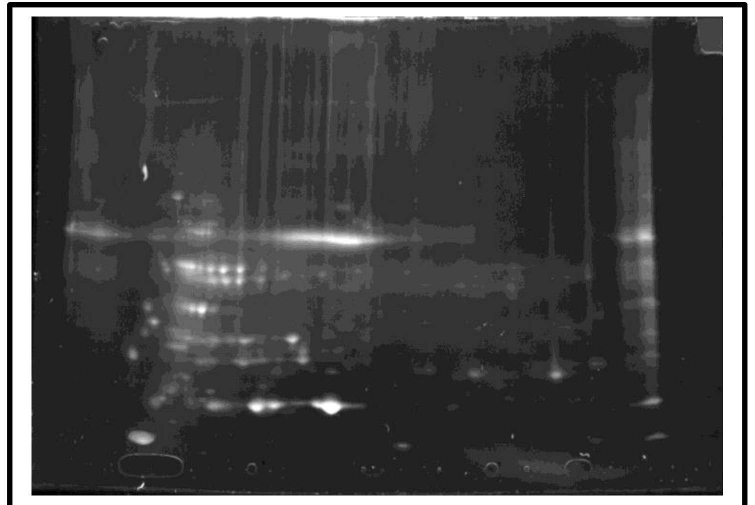






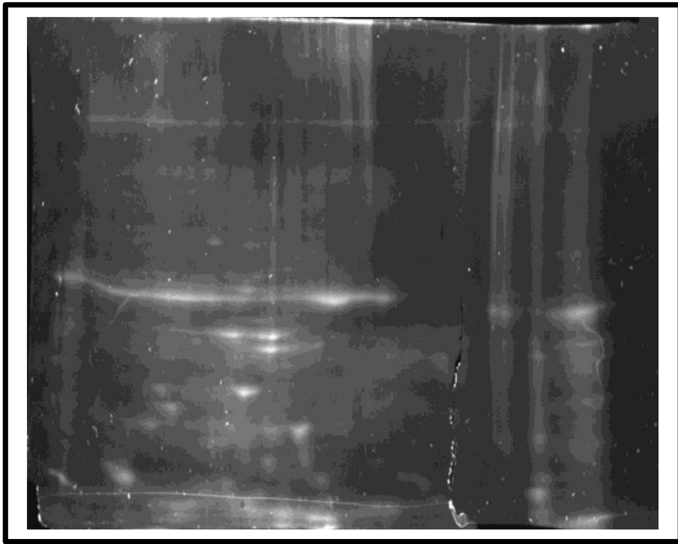


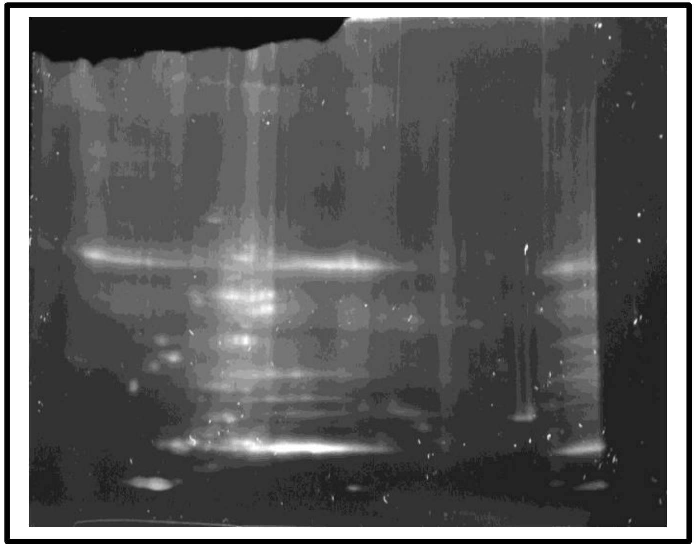


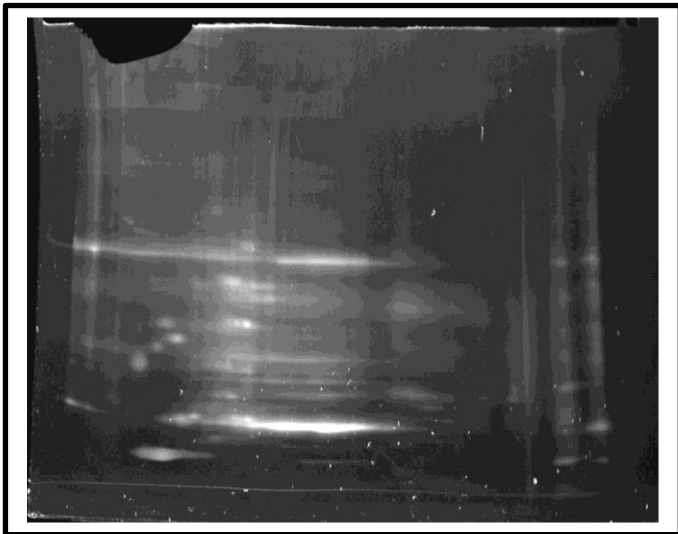


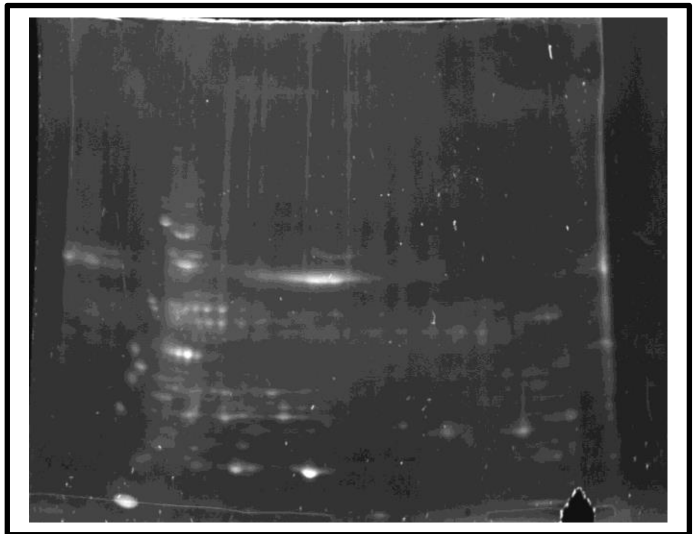
DAY 7 TUGELA SUSCEPTIBLE TEST GELS

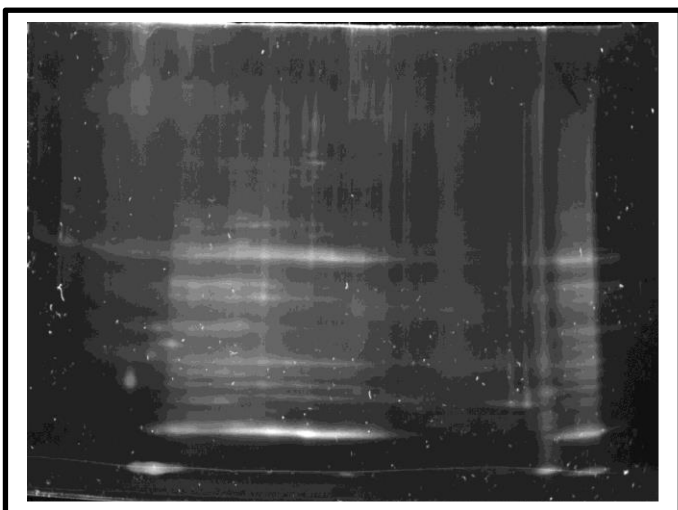
DAY 7 TUGELA SUSCEPTIBLE CONTROLS

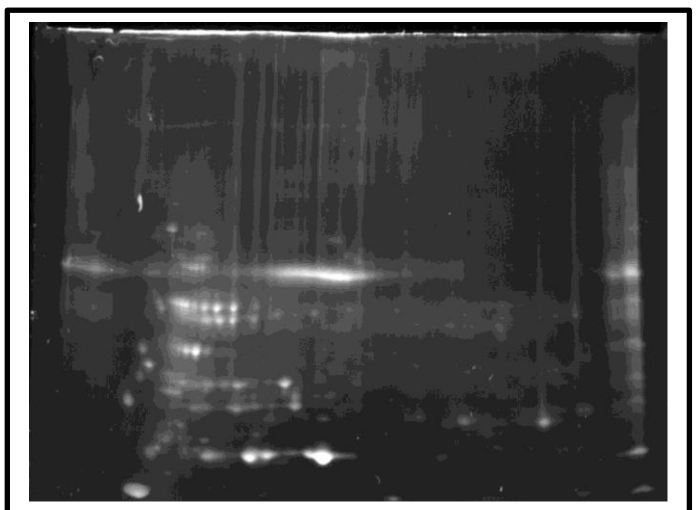












Proteins identified using ProteinPilot^{TM} software

Protein spot 1

Protein spot 2

Protein spot 3

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

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

Protein Group 4 - Phosphoglycerate kinase, chloroplastic OS=Triticum aestivum PE=2 SV=1

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

Protein spot 4

Proteins Detected

N	Unused	Total	% Cov (95)	Accession #	Name	Species	Peptides(95%)	Gene Ontology	Pathway	Int	Function
2	36.60	35.60	46.3	sp|P26302|KPPR_WHEAT	Phosphoribulokinase, chloroplastic OS=Triticum aestivum PE=2 SV=1	WHEAT	30	chloroplast; ATP bin...	Carboh...		
4	33.48	33.56	42.9	sp|P12782|PGKH_WHEAT	Phosphoglycerate kinase, chloroplastic OS=Triticum aestivum PE=2 SV=1	WHEAT	22	chloroplast; ATP bin...	Carboh...		
5	18.89	18.89	27.7	sp|P46285|S17P_WHEAT	Sedoheptulose-1,7-bisphosphatase, chloroplastic OS=Triticum aestivum PE=2 SV=1	WHEAT	11	chloroplast; fructose...	Carboh...		
6	12.59	12.59	13.5	sp|P20858|ATPB_WHEAT	ATP synthase subunit beta, chloroplastic OS=Triticum aestivum GN=atpB PE=3 SV=1	WHEAT	6	chloroplast thylakoid...			Produces ATP from ADP in the presence of a...
8	8.73	8.73	9.2	sp|P11383|RBL_WHEAT	Ribulose bisphosphate carboxylase large chain OS=Triticum aestivum GN=rbcL PE=1 SV=2	WHEAT	4	chloroplast; magnesi...			RuBisCO catalyzes two reactions: the carboxyl...
10	7.49	7.52	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	7.14	7.26	21.9	sp|P80602|BAS1_WHEAT	2-Cys peroxiredoxin BAS1, chloroplastic (Fragment) OS=Triticum aestivum GN=TSA PE=1 SV=2	WHEAT	4	chloroplast; peroxida...			May be an antioxidant enzyme particularly in th...
12	6.46	6.50	7.6	sp|P09195|F16P1_WHEAT	Fructose-1,6-bisphosphatase, chloroplastic OS=Triticum aestivum GN=FBP PE=2 SV=1	WHEAT	3	chloroplast; fructose...	Carboh...		
14	4.81	4.82	7.7	sp|P27665|PSBO_WHEAT	Oxygen-evolving enhancer protein 1, chloroplastic OS=Triticum aestivum GN=PSBO PE=2 SV=1	WHEAT	3	chloroplast thylakoid...			Stabilizes the manganese cluster which is the...
15	4.26	4.27	6.0	sp|P08823|RUBA_WHEAT	RuBisCO large subunit-binding protein subunit alpha, chloroplastic (Fragment) OS=Triticum aestivum PE=1 SV=1	WHEAT	2	chloroplast; ATP bin...			This protein binds RuBisCO small and large su...
16	4.25	4.27	9.1	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 pl...
19	2.00	5.46	13.2	sp|P00871|RBS1_WHEAT	Ribulose bisphosphate carboxylase small chain PWS4.3, chloroplastic OS=Triticum aestivum PE=3 SV=2	WHEAT	4	chloroplast; monoox...			RuBisCO catalyzes two reactions: the carboxyl...
20	2.00	5.46	20.4	sp|P07398|RBS3_WHEAT	Ribulose bisphosphate carboxylase small chain clone 512 (Fragment) OS=Triticum aestivum PE=2 SV=1	WHEAT	4	chloroplast; monoox...			RuBisCO catalyzes two reactions: the carboxyl...
23	2.00	2.00	2.6	sp|Q36814|PSBD_WHEAT	Photosystem II D2 protein OS=Triticum aestivum GN=psbD PE=3 SV=2	WHEAT	1	chloroplast thylakoid...			Photosystem II (PSII) is a light-driven water pl...
25	1.87	1.97	6.1	sp|Q9ZP21|TRXM_WHEAT	Thioredoxin M-type, chloroplastic OS=Triticum aestivum PE=2 SV=1	WHEAT	1	chloroplast; protein d...			Participates in various redox reactions through...
7	11.03	11.03	39.0	cont|000143	pdb|1FNI_A| A Chain A, Crystal Structure Of Porcine Beta Trypsin With 0.01% Polydocanol [Sus scrofa (contaminant)]	Sus scrofa (contaminant)	19				
13	6.00	6.00	10.5	tr|A0A0C4ERA6|A0A0C4ERA6...	Uncharacterized protein OS=Puccinia triticina (isolate 1-1 / race 1 (BBBD)) GN=PTTG_03323 PE=3 SV=1	PUCT1	3				
18	3.51	3.57	1.3	tr|A0A0C4FAC4|A0A0C4FAC4...	Uncharacterized protein OS=Puccinia triticina (isolate 1-1 / race 1 (BBBD)) GN=PTTG_10165 PE=4 SV=1	PUCT1	4				
22	2.00	2.00	0.8	tr|A0A0C4EYX2|A0A0C4EYX2...	Uncharacterized protein OS=Puccinia triticina (isolate 1-1 / race 1 (BBBD)) GN=PTTG_06027 PE=4 SV=1	PUCT1	1				
24	1.96	2.00	1.3	tr|A0A0C4ENG6|A0A0C4ENG...	Uncharacterized protein OS=Puccinia triticina (isolate 1-1 / race 1 (BBBD)) GN=PTTG_02311 PE=4 SV=1	PUCT1	1				
26	1.40	1.46	2.5	tr|A0A0C4F6N7|A0A0C4F6N7...	Uncharacterized protein OS=Puccinia triticina (isolate 1-1 / race 1 (BBBD)) GN=PTTG_08807 PE=4 SV=1	PUCT1	1				
27	1.34	1.41	1.2	tr|A0A0C4EMQ2|A0A0C4EMQ...	Uncharacterized protein OS=Puccinia triticina (isolate 1-1 / race 1 (BBBD)) GN=PTTG_02042 PE=4 SV=1	PUCT1	1				
1	42.54	42.54	28.7	cont|000135	cra|hCP1609934.2| keratin 1 (epidermolytic hyperkeratosis) [Homo sapiens (contaminant)]	Homo sapiens (contaminant)	25				
3	34.80	34.91	23.9	cont|000136	cra|hCP1812061| keratin 10 (epidermolytic hyperkeratosis; keratosis palmaris et plantaris) [Homo sapiens (contaminant)]	Homo sapiens (contaminant)	20				
9	7.60	11.80	13.3	cont|000127	spt|P48668| Keratin, type II cytoskeletal 6E (Cytokeratin 6E) (CK 6E) (K6e keratin) [Homo sapiens (contaminant)]	Homo sapiens (contaminant)	8				
17	3.69	3.76	12.7	cont|000124	spt|P81605| Dermcidin precursor (Preproteolysin) (Contains: Survival-promoting peptide DCD-1) [Homo sapiens (cont...	Homo sapiens (contaminant)	2				
21	2.00	2.00	3.8	cont|000156	gi|68566311|sp|P0A6N1|EFTU_ECOLI Elongation factor Tu (EF-Tu) (P-43) [Escherichia coli (contaminant)]	Escherichia coli (contamin...	1				

Protein Group 5 - Sedoheptulose-1,7-bisphosphatase, chloroplastic OS=Triticum aestivum PE=2 SV=1

Proteins in Group

N	Unused	Total	Accessio...	Name	Species
5	18.89	18.89	sp|P4628...	Sedoheptulose-1,7-b...	WHEAT

Peptides in Group

Peptide Locus	Con...	Conf	Sequence	Modifications	Protein Modifications	Cleavages	ΔMass	Obs MW	z	Sc	Spectrum	Type
158.001	2.00	99	FEETLYGSSR				0.0001	1187.54...	2	9	1.1.1.79.14	Winner
154.001	2.00	99	GIFTNVTSPTAK				-0.0016	1234.65...	2	11	1.1.1.87.13	Winner
155.001	2.00	99	LLFEVAPLGF			cleaved F-L...	-0.0022	1104.61...	2	10	1.1.1.230.4	Winner
147.001	2.00	99	LTGVTGGDQVAAAMGIYGPR	Oxidation(M)@14			0.0014	1948.96...	3	16	1.1.1.107.10	Winner
153.001	2.00	99	LVNYYVK				0.0036	897.4997	2	8	1.1.1.72.5	Winner

Protein Sequence Coverage - Sedoheptulose-1,7-bisphosphatase, chloroplastic OS=Triticum aestivum PE=2 SV=1

Proteins Detected

N	Unused	Total	% Cov (95)	Accession #	Name	Species	Peptides(95%)	Gene Ontology	Pathway	Int	Function
2	36.60	35.60	46.3	sp|P26302|KPPR_WHEAT	Phosphoribulokinase, chloroplastic OS=Triticum aestivum PE=2 SV=1	WHEAT	30	chloroplast; ATP bin...	Carboh...		
4	33.48	33.56	42.9	sp|P12782|PGKH_WHEAT	Phosphoglycerate kinase, chloroplastic OS=Triticum aestivum PE=2 SV=1	WHEAT	22	chloroplast; ATP bin...	Carboh...		
5	18.89	18.89	27.7	sp|P46285|S17P_WHEAT	Sedoheptulose-1,7-bisphosphatase, chloroplastic OS=Triticum aestivum PE=2 SV=1	WHEAT	11	chloroplast; fructose...	Carboh...		
6	12.59	12.59	13.5	sp|P20858|ATPB_WHEAT	ATP synthase subunit beta, chloroplastic OS=Triticum aestivum GN=atpB PE=3 SV=1	WHEAT	6	chloroplast thylakoid...			Produces ATP from ADP in the presence of a...
8	8.73	8.73	9.2	sp|P11383|RBL_WHEAT	Ribulose bisphosphate carboxylase large chain OS=Triticum aestivum GN=rbcL PE=1 SV=2	WHEAT	4	chloroplast; magnesi...			RuBisCO catalyzes two reactions: the carboxyl...
10	7.49	7.52	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	7.14	7.26	21.9	sp|P80602|BAS1_WHEAT	2-Cys peroxiredoxin BAS1, chloroplastic (Fragment) OS=Triticum aestivum GN=TSA PE=1 SV=2	WHEAT	4	chloroplast; peroxida...			May be an antioxidant enzyme particularly in th...
12	6.46	6.50	7.6	sp|P09195|F16P1_WHEAT	Fructose-1,6-bisphosphatase, chloroplastic OS=Triticum aestivum GN=FBP PE=2 SV=1	WHEAT	3	chloroplast; fructose...	Carboh...		
14	4.81	4.82	7.7	sp|P27665|PSBO_WHEAT	Oxygen-evolving enhancer protein 1, chloroplastic OS=Triticum aestivum GN=PSBO PE=2 SV=1	WHEAT	3	chloroplast thylakoid...			Stabilizes the manganese cluster which is the...
15	4.26	4.27	6.0	sp|P08823|RUBA_WHEAT	RuBisCO large subunit-binding protein subunit alpha, chloroplastic (Fragment) OS=Triticum aestivum PE=1 SV=1	WHEAT	2	chloroplast; ATP bin...			This protein binds RuBisCO small and large su...
16	4.25	4.27	9.1	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 pl...
19	2.00	5.46	13.2	sp|P00871|RBS1_WHEAT	Ribulose bisphosphate carboxylase small chain PWS4.3, chloroplastic OS=Triticum aestivum PE=3 SV=2	WHEAT	4	chloroplast; monoox...			RuBisCO catalyzes two reactions: the carboxyl...
20	2.00	5.46	20.4	sp|P07398|RBS3_WHEAT	Ribulose bisphosphate carboxylase small chain clone 512 (Fragment) OS=Triticum aestivum PE=2 SV=1	WHEAT	4	chloroplast; monoox...			RuBisCO catalyzes two reactions: the carboxyl...
23	2.00	2.00	2.6	sp|Q36814|PSBD_WHEAT	Photosystem II D2 protein OS=Triticum aestivum GN=psbD PE=3 SV=2	WHEAT	1	chloroplast thylakoid...			Photosystem II (PSII) is a light-driven water pl...
25	1.87	1.97	6.1	sp|Q9ZP21|TRXM_WHEAT	Thioredoxin M-type, chloroplastic OS=Triticum aestivum PE=2 SV=1	WHEAT	1	chloroplast; protein d...			Participates in various redox reactions through...
7	11.03	11.03	39.0	cont|000143	pdb|1FNI_A| A Chain A, Crystal Structure Of Porcine Beta Trypsin With 0.01% Polydocanol [Sus scrofa (contaminant)]	Sus scrofa (contaminant)	19				
13	6.00	6.00	10.5	tr|A0A0C4ERA6|A0A0C4ERA6...	Uncharacterized protein OS=Puccinia triticina (isolate 1-1 / race 1 (BBBD)) GN=PTTG_03323 PE=3 SV=1	PUCT1	3				
18	3.51	3.57	1.3	tr|A0A0C4FAC4|A0A0C4FAC4...	Uncharacterized protein OS=Puccinia triticina (isolate 1-1 / race 1 (BBBD)) GN=PTTG_10165 PE=4 SV=1	PUCT1	4				
22	2.00	2.00	0.8	tr|A0A0C4EYX2|A0A0C4EYX2...	Uncharacterized protein OS=Puccinia triticina (isolate 1-1 / race 1 (BBBD)) GN=PTTG_06027 PE=4 SV=1	PUCT1	1				
24	1.96	2.00	1.3	tr|A0A0C4ENG6|A0A0C4ENG...	Uncharacterized protein OS=Puccinia triticina (isolate 1-1 / race 1 (BBBD)) GN=PTTG_02311 PE=4 SV=1	PUCT1	1				
26	1.40	1.46	2.5	tr|A0A0C4F6N7|A0A0C4F6N7...	Uncharacterized protein OS=Puccinia triticina (isolate 1-1 / race 1 (BBBD)) GN=PTTG_08807 PE=4 SV=1	PUCT1	1				
27	1.34	1.41	1.2	tr|A0A0C4EMQ2|A0A0C4EMQ...	Uncharacterized protein OS=Puccinia triticina (isolate 1-1 / race 1 (BBBD)) GN=PTTG_02042 PE=4 SV=1	PUCT1	1				
1	42.54	42.54	28.7	cont|000135	cra|hCP1609934.2| keratin 1 (epidermolytic hyperkeratosis) [Homo sapiens (contaminant)]	Homo sapiens (contaminant)	25				
3	34.80	34.91	23.9	cont|000136	cra|hCP1812061| keratin 10 (epidermolytic hyperkeratosis; keratosis palmaris et plantaris) [Homo sapiens (contaminant)]	Homo sapiens (contaminant)	20				
9	7.60	11.80	13.3	cont|000127	spt|P48668| Keratin, type II cytoskeletal 6E (Cytokeratin 6E) (CK 6E) (K6e keratin) [Homo sapiens (contaminant)]	Homo sapiens (contaminant)	8				
17	3.69	3.76	12.7	cont|000124	spt|P81605| Dermcidin precursor (Preproteolysin) (Contains: Survival-promoting peptide DCD-1) [Homo sapiens (cont...	Homo sapiens (contaminant)	2				
21	2.00	2.00	3.8	cont|000156	gi|68566311|sp|P0A6N1|EFTU_ECOLI Elongation factor Tu (EF-Tu) (P-43) [Escherichia coli (contaminant)]	Escherichia coli (contamin...	1				

Protein Group 6 - ATP synthase subunit beta, chloroplastic OS=Triticum aestivum GN=atpB PE=3 SV=1

Proteins in Group

N	Unused	Total	Accessio...	Name	Species
6	12.59	12.59	sp|P2085...	ATP synthase subun...	WHEAT

Peptides in Group

Peptide Locus	Con...	Conf	Sequence	Modifications	Protein Modifications	Cleavages	ΔMass	Obs MW	z	Sc	Spectrum	Type
159.001	2.00	99	AVAMSATDGLMR	Oxidation(M)@4 Oxidation(M)@11			0.0203	1253.59...	2	10	1.1.1.47.9	Winner
165.001	2.00	99	IGLFGGAGVGK				-0.0027	974.5522	2	12	1.1.1.101.5	Winner
163.001	2.00	99	LSIFETGIK				0.0018	1006.57...	2	9	1.1.1.117.3	Winner
162.001	2.00	99	SAPAFIELDTK				-0.0027	1190.61...	2	9	1.1.1.109.6	Winner

Protein Sequence Coverage - ATP synthase subunit beta, chloroplastic OS=Triticum aestivum GN=atpB PE=3 SV=1

Protein spot 5

Protein spot 6

ProteinPilot™ Software - [Result - E:\Adrian LCMS data\Adrian_4801.group]

File Configure Window Help

Workflow Tasks

Identify Proteins
- LC...
- Spot-Based (MS only)...
- Spot-Based (MS and MS/MS)...

View
- Analysis Log...
- Result...

Export
- Peptide Summary...
- Distinct Peptide Summary...
- Protein Summary...
- Spectrum Summary...
- MGF Peaklist(s)...
- mzIdentML...
- Features...

Protein ID | Features | Spectra | Summary Statistics

Proteins Detected

N	Unused	Total	% Cov (95)	Accession #	Name	Species	Peptides(95%)	Gene Ontology	Pathway	Int	Function
1	51.01	51.01	49.6	sp|P12782|PGKH_WHEAT	Phosphoglycerate kinase, chloroplastic OS=Triticum aestivum PE=2 SV=1	WHEAT	35	chloroplast; ATP bin...	Carboh...		
3	22.00	22.00	29.2	sp|P26302|KPPR_WHEAT	Phosphoribulokinase, chloroplastic OS=Triticum aestivum PE=2 SV=1	WHEAT	11	chloroplast; ATP bin...	Carboh...		
4	14.24	14.24	20.1	sp|P20858|ATPB_WHEAT	ATP synthase subunit beta, chloroplastic OS=Triticum aestivum GN=atpB PE=3 SV=1	WHEAT	8	chloroplast thylakoid...			Produces ATP from ADP in the presence of a...
6	8.98	8.98	12.2	sp|P46285|S17P_WHEAT	Sedoheptulose-1,7-bisphosphatase, chloroplastic OS=Triticum aestivum PE=2 SV=1	WHEAT	5	chloroplast; fructose...	Carboh...		
7	8.00	8.00	31.0	sp|P07398|RBS3_WHEAT	Ribulose bisphosphate carboxylase small chain clone 512 (Fragment) OS=Triticum aestivum PE=2 SV=1	WHEAT	5	chloroplast; monoox...			RuBisCO catalyzes two reactions: the carboxyl...
10	5.33	5.46	7.1	sp|P11383|RBL_WHEAT	Ribulose bisphosphate carboxylase large chain OS=Triticum aestivum GN=rbcL PE=1 SV=2	WHEAT	3	chloroplast; magnesi...			RuBisCO catalyzes two reactions: the carboxyl...
11	3.71	3.71	6.6	sp|P27665|PSBO_WHEAT	Oxygen-evolving enhancer protein 1, chloroplastic OS=Triticum aestivum GN=PSBO PE=2 SV=1	WHEAT	2	chloroplast thylakoid...			Stabilizes the manganese cluster which is the...
12	2.38	2.39	3.3	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 th...
13	2.18	2.18	7.6	sp|B0LXM0|METK_WHEAT	S-adenosylmethionine synthase OS=Triticum aestivum GN=SAMS PE=2 SV=1	WHEAT	2	cytoplasm; ATP bind...	Amino-...		Catalyzes the formation of S-adenosylmethioni...
14	2.00	6.00	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...
17	2.00	2.00	3.1	sp|P12463|PSBA_WHEAT	Photosystem II protein D1 OS=Triticum aestivum GN=psbA PE=3 SV=2	WHEAT	1	chloroplast thylakoid...			Photosystem II (PSII) is a light-driven water:pl...
18	1.86	6.08	13.1	sp|P26667|RBS2_WHEAT	Ribulose bisphosphate carboxylase small chain PW9, chloroplastic OS=Triticum aestivum PE=3 SV=1	WHEAT	3	chloroplast; monoox...			RuBisCO catalyzes two reactions: the carboxyl...
9	6.04	6.04	17.9	cont|000143	pdb|1FNI_A| A Chain A, Crystal Structure Of Porcine Beta Trypsin With 0.01% Polydocanol [Sus scrofa (contaminant)]	Sus scrofa (contaminant)	12				
8	6.09	6.09	10.5	tr|A0A0C4ERA6|A0A0C4ERA6...	Uncharacterized protein OS=Puccinia triticina (isolate 1-1 / race 1 (BBBD)) GN=PTTG_03323 PE=3 SV=1	PUCT1	3				
15	2.00	2.00	3.6	tr|A0A0C4EYH7|A0A0C4EYH7...	Uncharacterized protein OS=Puccinia triticina (isolate 1-1 / race 1 (BBBD)) GN=PTTG_05877 PE=3 SV=1	PUCT1	1				
2	25.50	25.50	18.6	cont|000135	cra|hCP1609934.2| keratin 1 (epidermolytic hyperkeratosis) [Homo sapiens (contaminant)]	Homo sapiens (contaminant)	13				
5	11.83	11.83	7.6	cont|000136	cra|hCP1812061| keratin 10 (epidermolytic hyperkeratosis; keratosis palmaris et plantaris) [Homo sapiens (contaminant)]	Homo sapiens (contaminant)	5				
16	2.00	2.00	12.7	cont|000124	spt|P81605| Dermcidin precursor (Preproteolysin) (Contains: Survival-promoting peptide DCD-1) [Homo sapiens (cont...	Homo sapiens (contaminant)	1				

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	51.01	51.01	sp|P1278...	Phosphoglycerate kin...	WHEAT

Peptides in Group

Peptide Locus	Con...	Conf	Sequence	Modifications	Protein Modifications	Cleavages	ΔMass	Obs MW	z	Sc	Spectrum	Type
10.001	2.00	99	ADLNVPLDDNQNITDDTR				0.0031	2027.94...	3	12	1.1.1.106.10	Winner
29.001	2.00	99	AEDVIGPEVEK				0.0011	1184.59...	2	8	1.1.1.65.8	Winner
56.001	2.00	99	AKGVSLLLPSDVIIADK			missed K-...	-0.0042	1738.01...	3	8	1.1.1.132.3	Winner
69.001	2.00	75.1	ELPGVVALDEGVMTR	Oxidation(M)@13			-0.0015	1600.81...	3	9	1.1.1.123.5	Winner
52.001	2.00	99	FAAIVGGSK			cleaved P-...	-0.0036	848.4720	2	11	1.1.1.58.2	Winner
61.001	2.00	99	FAVGTESIAKK	Lys-loss@C-term		missed K-K...	-0.0010	1021.54...	2	12	1.1.1.72.3	Winner
47.001	2.00	99	FLKPSVAGF			cleaved F-L...	-0.0015	964.5367	2	10	1.1.1.108.5	Winner
46.001	2.00	99	FLKPSVAGFLLQK				0.0016	1446.86...	3	15	1.1.1.122.3	Winner
25.001	2.00	99	FSLAPLVPR				-0.0001	998.5912	2	11	1.1.1.128.3	Winner
39.001	2.00	99	FYKEEEKNDPEFAK			missed K-E...	0.0033	1772.82...	4	17	1.1.1.45.5	Winner
37.001	2.00	99	GAVLLLENVR			cleaved N-...	-0.0037	1082.64...	2	9	1.1.1.116.3	Winner
53.001	2.00	99	IGVIESLLEK				0.0023	1099.65...	2	16	1.1.1.137.4	Winner
28.001	2.00	99	KAEDVIGPEVEK			missed K-A...	0.0025	1312.68...	3	13	1.1.1.48.3	Winner
63.001	2.00	99	KGVTTIIGGGDSVAAVEK			missed K-...	-0.0008	1700.92...	3	23	1.1.1.78.9	Winner

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

[illegible]

10:19 PM 2016/01/17

ProteinPilot™ Software - [Result - E:\Adrian LCMS data\Adrian_4801.group]

File Configure Window Help

Workflow Tasks

Identify Proteins
- LC...
- Spot-Based (MS only)...
- Spot-Based (MS and MS/MS)...

View
- Analysis Log...
- Result...

Export
- Peptide Summary...
- Distinct Peptide Summary...
- Protein Summary...
- Spectrum Summary...
- MGF Peaklist(s)...
- mzIdentML...
- Features...

Protein ID | Features | Spectra | Summary Statistics

Proteins Detected

N	Unused	Total	% Cov (95)	Accession #	Name	Species	Peptides(95%)	Gene Ontology	Pathway	Int	Function
1	51.01	51.01	49.6	sp|P12782|PGKH_WHEAT	Phosphoglycerate kinase, chloroplastic OS=Triticum aestivum PE=2 SV=1	WHEAT	35	chloroplast; ATP bin...	Carboh...		
3	22.00	22.00	29.2	sp|P26302|KPPR_WHEAT	Phosphoribulokinase, chloroplastic OS=Triticum aestivum PE=2 SV=1	WHEAT	11	chloroplast; ATP bin...	Carboh...		
4	14.24	14.24	20.1	sp|P20858|ATPB_WHEAT	ATP synthase subunit beta, chloroplastic OS=Triticum aestivum GN=atpB PE=3 SV=1	WHEAT	8	chloroplast thylakoid...			Produces ATP from ADP in the presence of a...
6	8.98	8.98	12.2	sp|P46285|S17P_WHEAT	Sedoheptulose-1,7-bisphosphatase, chloroplastic OS=Triticum aestivum PE=2 SV=1	WHEAT	5	chloroplast; fructose...	Carboh...		
7	8.00	8.00	31.0	sp|P07398|RBS3_WHEAT	Ribulose bisphosphate carboxylase small chain clone 512 (Fragment) OS=Triticum aestivum PE=2 SV=1	WHEAT	5	chloroplast; monoox...			RuBisCO catalyzes two reactions: the carboxyl...
10	5.33	5.46	7.1	sp|P11383|RBL_WHEAT	Ribulose bisphosphate carboxylase large chain OS=Triticum aestivum GN=rbcL PE=1 SV=2	WHEAT	3	chloroplast; magnesi...			RuBisCO catalyzes two reactions: the carboxyl...
11	3.71	3.71	6.6	sp|P27665|PSBO_WHEAT	Oxygen-evolving enhancer protein 1, chloroplastic OS=Triticum aestivum GN=PSBO PE=2 SV=1	WHEAT	2	chloroplast thylakoid...			Stabilizes the manganese cluster which is the...
12	2.38	2.39	3.3	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 th...
13	2.18	2.18	7.6	sp|B0LXM0|METK_WHEAT	S-adenosylmethionine synthase OS=Triticum aestivum GN=SAMS PE=2 SV=1	WHEAT	2	cytoplasm; ATP bind...	Amino-...		Catalyzes the formation of S-adenosylmethioni...
14	2.00	6.00	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...
17	2.00	2.00	3.1	sp|P12463|PSBA_WHEAT	Photosystem II protein D1 OS=Triticum aestivum GN=psbA PE=3 SV=2	WHEAT	1	chloroplast thylakoid...			Photosystem II (PSII) is a light-driven water:pl...
18	1.86	6.08	13.1	sp|P26667|RBS2_WHEAT	Ribulose bisphosphate carboxylase small chain PW9, chloroplastic OS=Triticum aestivum PE=3 SV=1	WHEAT	3	chloroplast; monoox...			RuBisCO catalyzes two reactions: the carboxyl...
9	6.04	6.04	17.9	cont|000143	pdb|1FNI_A| A Chain A, Crystal Structure Of Porcine Beta Trypsin With 0.01% Polydocanol [Sus scrofa (contaminant)]	Sus scrofa (contaminant)	12				
8	6.09	6.09	10.5	tr|A0A0C4ERA6|A0A0C4ERA6...	Uncharacterized protein OS=Puccinia triticina (isolate 1-1 / race 1 (BBBD)) GN=PTTG_03323 PE=3 SV=1	PUCT1	3				
15	2.00	2.00	3.6	tr|A0A0C4EYH7|A0A0C4EYH7...	Uncharacterized protein OS=Puccinia triticina (isolate 1-1 / race 1 (BBBD)) GN=PTTG_05877 PE=3 SV=1	PUCT1	1				
2	25.50	25.50	18.6	cont|000135	cra|hCP1609934.2| keratin 1 (epidermolytic hyperkeratosis) [Homo sapiens (contaminant)]	Homo sapiens (contaminant)	13				
5	11.83	11.83	7.6	cont|000136	cra|hCP1812061| keratin 10 (epidermolytic hyperkeratosis; keratosis palmaris et plantaris) [Homo sapiens (contaminant)]	Homo sapiens (contaminant)	5				
16	2.00	2.00	12.7	cont|000124	spt|P81605| Dermcidin precursor (Preproteolysin) (Contains: Survival-promoting peptide DCD-1) [Homo sapiens (cont...	Homo sapiens (contaminant)	1				

Protein Group 4 - ATP synthase subunit beta, chloroplastic OS=Triticum aestivum GN=atpB PE=3 SV=1

Remove Redundancy

Proteins in Group

N	Unused	Total	Accessio...	Name	Species
4	14.24	14.24	sp|P2085...	ATP synthase subun...	WHEAT

Peptides in Group

Peptide Locus	Con...	Conf	Sequence	Modifications	Protein Modifications	Cleavages	ΔMass	Obs MW	z	Sc	Spectrum	Type
109.001	2.00	99	AVAMSATDGLMR	Oxidation(M)@4 Oxidation(M)@11			0.0003	1253.57...	2	7	1.1.1.44.15	Winner
118.001	2.00	99	DVNKQDVLLFIDNIFR			missed K-...	-0.0014	1948.04...	3	11	1.1.1.209.4	Winner
114.001	2.00	99	IGLFGGAGVGK				-0.0015	974.5536	2	10	1.1.1.99.4	Winner
121.001	2.00	99	IVGNEHYETAQR				0.0023	1415.68...	3	11	1.1.1.34.5	Winner
112.001	2.00	99	LSIFETGIK				0.0004	1006.57...	2	8	1.1.1.114.3	Winner
111.001	2.00	99	SAPAFIELDTK				-0.0009	1190.61...	2	10	1.1.1.100.7	Winner
117.001	2.00	99	VALVYGQMNEPPGAR	Oxidation(M)@8			-0.0007	1616.78...	3	10	1.1.1.78.7	Winner
113.001	0.16	43.2	VVDLLAPYR				-0.0043	1044.59...	2	6	1.1.1.123.3	Winner
119.001	0.07	99	FVQAGSEVSALLGR	Cation:Na(E)@7			-0.0056	1454.74...	3	11	1.1.1.130.2	Winner
116.001	0.01	3.2	TREGNDLYMEMK	Oxidation(M)@9 Oxidation(M)@11		missed R-E...	-0.0035	1517.64...	3	4	1.1.1.34.7	Winner
110.001	0.01	2.2	GMEVIDTGAPLSVPVGGATLGR	Oxidation(M)@2			-0.0003	2112.08...	3	4	1.1.1.130.6	Winner
115.001	0.00	1.8	AHGGVSVFGGVGER				-0.0065	1327.65...	3	5	1.1.1.63.8	Winner
107.001	0.00	0.3	LPYIYNALVVQSR				-0.0009	1534.84...	3	4	1.1.1.145.2	Winner

Protein Sequence Coverage - ATP synthase subunit beta, chloroplastic OS=Triticum aestivum GN=atpB PE=3 SV=1

[illegible]

10:19 PM 2016/01/17

Protein spot 7

Protein spot 8

Protein spot 9

Protein spot 10

ProteinPilot™ Software - [Result - E:\Adrian LCMS data\Adrian_1.group]

File Configure Window Help

Workflow Tasks: Identify Proteins; LC...; Spot-Based (MS only)...; Spot-Based (MS and MS/MS)...; View; Analysis Log...; Result...; Export; Peptide Summary...; Distinct Peptide Summary...; Protein Summary...; Spectrum Summary...; MGF Peaklist(s)...; mzIdentML...; Features...

Protein ID | Features | Spectra | Summary Statistics

Proteins Detected

N	Unused	Total	% Cov (...)	Accession #	Name	Species	Peptides(95%)	Gene Ontology	Pathway	Int	Function
1	42.75	42.75	29.0	cont|000135	cra|hCP1609934.2| keratin 1 (epidermolytic hyperkeratosis) [Homo sapiens (contaminant)]	Homo sapiens (contaminant)	26				
4	6.73	6.73	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)	13				
2	30.11	30.11	18.6	cont|000136	cra|hCP1812061| keratin 10 (epidermolytic hyperkeratosis; keratosis palmaris et plantaris) [Homo sapiens (contaminant)]	Homo sapiens (contaminant)	14				
6	4.08	4.09	13.3	sp|P07398|RBS3_WHEAT	Ribulose bisphosphate carboxylase small chain clone 512 (Fragment) OS=Triticum aestivum PE=2 SV=1	WHEAT	2	chloroplast; monoox...			RuBisCO catalyzes two reactions: the carboxyl...
7	4.00	4.00	12.7	cont|000124	sp|P81605| Dermcidin precursor (Preproteolysin) (Contains: Survival-promoting peptide; DCD-1) [Homo sapiens (cont...	Homo sapiens (contaminant)	2				
3	8.67	11.61	9.1	cont|000126	sp|P48668| Keratin, type II cytoskeletal 6C (Cytokeratin 6C) (CK 6C) (K6c keratin) [Homo sapiens (contaminant)]	Homo sapiens (contaminant)	5				
9	2.00	4.09	8.6	sp|P00871|RBS1_WHEAT	Ribulose bisphosphate carboxylase small chain PWS4.3, chloroplastic OS=Triticum aestivum PE=3 SV=2	WHEAT	2	chloroplast; monoox...			RuBisCO catalyzes two reactions: the carboxyl...
10	2.00	2.00	7.1	tr|A0A0C4EZZ1|A0A0C4EZZ1...	Uncharacterized protein OS=Puccinia triticina (isolate 1-1 / race 1 (BBBD)) GN=PTTG_06406 PE=4 SV=1	PUCT1	1				
5	5.13	5.13	4.6	sp|P11383|RBL_WHEAT	Ribulose bisphosphate carboxylase large chain OS=Triticum aestivum GN=rbcL PE=1 SV=2	WHEAT	2	chloroplast; magnesi...			RuBisCO catalyzes two reactions: the carboxyl...
11	2.00	2.00	3.8	tr|A0A0C4F7U7|A0A0C4F7U7...	Uncharacterized protein OS=Puccinia triticina (isolate 1-1 / race 1 (BBBD)) GN=PTTG_09231 PE=4 SV=1	PUCT1	1				
8	2.19	2.22	1.8	sp|P12112|ATPA_WHEAT	ATP synthase subunit alpha, chloroplastic OS=Triticum aestivum GN=atpA PE=3 SV=2	WHEAT	1	chloroplast thylakoid...			Produces ATP from ADP in the presence of a...

Protein Group 6 - Ribulose bisphosphate carboxylase small chain clone 512 (Fragment) OS=Triticum aestivum PE=2 SV=1

☑ Remove Redundancy

Proteins in Group

N	Unused	Total	Accessio...	Name	Species
6	4.08	4.09	sp|P0739...	Ribulose bisphosph...	WHEAT
9	2.00	4.09	sp|P0087...	Ribulose bisphosph...	WHEAT
	0.03	2.12	sp|P2666...	Ribulose bisphosphat...	WHEAT

Peptides in Group

Peptide Locus	Con...	Conf	Sequence	Modifications	Protein Modifications	Cleavages	ΔMass	Obs MW	z	Sc	Spectrum	Type
141.001	2.00	99	IIGFDNMR	Oxidation(M)@7			-0.0069	980.4681	2	8	1.1.1.76.7	Winner
138.001	2.00	99	QVDYLIR				-0.0082	905.4889	2	8	1.1.1.86.4	Winner
140.001	0.08	33.2	EYPDAYVR				-0.0105	1011.45...	2	4	1.1.1.73.6	Winner
139.001	0.00	0.6	PMFGCTDATQVINEVEEVKK	Carbamidomethyl(C)@5 Deamidated(Q)@10 Deamidated(N)@13		cleaved L-...	0.1104	2296.17...	3	4	1.1.1.139.5	Winner
177.004	0.00	0.7	YLPPLSTEALLK	Oxidation(P)@3 Oxidation(P)@4		cleaved A-Y...	-0.0276	1375.73...	2	4	1.1.1.166.6	Winner
155.001	2.00	99	VIGFDNLR				-0.0073	932.5007	2	8	1.1.1.101.3	Other
361.001	0.02	7.8	KPETLSYLPPLSTEALLK			missed K-F...	-0.0168	2049.12...	3	4	1.1.1.160.4	Other
180.002	0.00	8.1	VIGFDNMR	Oxidation(M)@7			-0.0080	966.4513	2	4	1.1.1.66.8	Other
154.001	0.00	0.6	PMFGCTDATQVLNEVEEVKK	Carbamidomethyl(C)@5 Deamidated(Q)@10 Deamidated(N)@13		cleaved L-P...	0.1104	2296.17...	3	4	1.1.1.139.5	Other
153.001	0.00	0.1	SRGSLGSVSNGGR			missed R-G...	0.1279	1232.75...	2	5	1.1.1.304.7	Other

Protein Sequence Coverage - Ribulose bisphosphate carboxylase small chain clone 512 (Fragment) OS=Triticum aestivum PE=2 SV=1

AYLPPLSTEALLKQVDYLIRSKWVPCLEFSKVGFIFREHNASPGYYDGRYWTMWKLPMFGCTDATQVINEVEEVKKEYPDAYVRIIGFDNMRQVQCVSFIAFKPPGCEESGKA

08:25 AM 2016/01/12

ProteinPilot™ Software - [Result - E:\Adrian LCMS data\Adrian_1.group]

File Configure Window Help

Workflow Tasks: Identify Proteins; LC...; Spot-Based (MS only)...; Spot-Based (MS and MS/MS)...; View; Analysis Log...; Result...; Export; Peptide Summary...; Distinct Peptide Summary...; Protein Summary...; Spectrum Summary...; MGF Peaklist(s)...; mzIdentML...; Features...

Protein ID | Features | Spectra | Summary Statistics

Proteins Detected

N	Unused	Total	% Cov (...)	Accession #	Name	Species	Peptides(95%)	Gene Ontology	Pathway	Int	Function
1	42.75	42.75	29.0	cont|000135	cra|hCP1609934.2| keratin 1 (epidermolytic hyperkeratosis) [Homo sapiens (contaminant)]	Homo sapiens (contaminant)	26				
4	6.73	6.73	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)	13				
2	30.11	30.11	18.6	cont|000136	cra|hCP1812061| keratin 10 (epidermolytic hyperkeratosis; keratosis palmaris et plantaris) [Homo sapiens (contaminant)]	Homo sapiens (contaminant)	14				
6	4.08	4.09	13.3	sp|P07398|RBS3_WHEAT	Ribulose bisphosphate carboxylase small chain clone 512 (Fragment) OS=Triticum aestivum PE=2 SV=1	WHEAT	2	chloroplast; monoox...			RuBisCO catalyzes two reactions: the carboxyl...
7	4.00	4.00	12.7	cont|000124	sp|P81605| Dermcidin precursor (Preproteolysin) (Contains: Survival-promoting peptide; DCD-1) [Homo sapiens (cont...	Homo sapiens (contaminant)	2				
3	8.67	11.61	9.1	cont|000126	sp|P48668| Keratin, type II cytoskeletal 6C (Cytokeratin 6C) (CK 6C) (K6c keratin) [Homo sapiens (contaminant)]	Homo sapiens (contaminant)	5				
9	2.00	4.09	8.6	sp|P00871|RBS1_WHEAT	Ribulose bisphosphate carboxylase small chain PWS4.3, chloroplastic OS=Triticum aestivum PE=3 SV=2	WHEAT	2	chloroplast; monoox...			RuBisCO catalyzes two reactions: the carboxyl...
10	2.00	2.00	7.1	tr|A0A0C4EZZ1|A0A0C4EZZ1...	Uncharacterized protein OS=Puccinia triticina (isolate 1-1 / race 1 (BBBD)) GN=PTTG_06406 PE=4 SV=1	PUCT1	1				
5	5.13	5.13	4.6	sp|P11383|RBL_WHEAT	Ribulose bisphosphate carboxylase large chain OS=Triticum aestivum GN=rbcL PE=1 SV=2	WHEAT	2	chloroplast; magnesi...			RuBisCO catalyzes two reactions: the carboxyl...
11	2.00	2.00	3.8	tr|A0A0C4F7U7|A0A0C4F7U7...	Uncharacterized protein OS=Puccinia triticina (isolate 1-1 / race 1 (BBBD)) GN=PTTG_09231 PE=4 SV=1	PUCT1	1				
8	2.19	2.22	1.8	sp|P12112|ATPA_WHEAT	ATP synthase subunit alpha, chloroplastic OS=Triticum aestivum GN=atpA PE=3 SV=2	WHEAT	1	chloroplast thylakoid...			Produces ATP from ADP in the presence of a...

Protein Group 8 - 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
8	2.19	2.22	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
147.001	2.00	99	GIALNLESK				-0.0030	943.5309	2	8	1.1.1.84.4	Winner
149.001	0.19	46.7	LIESPAPSIISR				-0.0136	1281.71...	2	5	1.1.1.100.6	Winner
146.001	0.00	0.2	VVQVGDGIAR				-0.0065	1012.56...	2	3	1.1.1.61.7	Winner
238.010	0.00	0.2	AMKQVAGK	Delta:H(2)C(2)(K)@3		missed K-...	-0.0216	857.4577	2	5	1.1.1.51.2	Winner
238.011	0.00	0.1	AMKQVAGK	Delta:H(2)C(2)@N-term		missed K-...	-0.0216	857.4577	2	5	1.1.1.51.2	Winner
208.003	0.00	12.4	ELIIGDR				-0.0070	814.4479	2	6	1.1.1.73.2	Winner
148.001	0.00	0.1	GIALNLESK	Deamidated(N)@5			-0.0076	944.5103	2	7	1.1.1.84.4	Winner
147.002	0.00	0.3	IALNLESK	Carbamidomethyl@N-term		cleaved G-I...	-0.0030	943.5309	2	7	1.1.1.84.4	Winner
173.013	0.00	0.4	INVGISVSR			cleaved A-I...	-0.0183	943.5269	2	8	1.1.1.63.6	Winner
149.002	0.00	1.2	LIESPAPSIISRR	Arg-loss@C-term		missed R-R...	-0.0136	1281.71...	2	5	1.1.1.100.6	Winner
150.001	0.00	0.3	TSQNQLAR	Deamidated(N)@4			0.0182	917.4749	2	3	1.1.1.51.4	Winner
152.001	0.00	0.2	TSQNQLAR	Deamidated(Q)@3			0.0182	917.4749	2	3	1.1.1.51.4	Winner
151.001	0.00	0.2	TSQNQLAR	Deamidated(Q)@5			0.0182	917.4749	2	3	1.1.1.51.4	Winner

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

MATLRVDEIHKILRERIEQYNRKVGIENIGRVVQVGDGIARIIGLGEIMSGELVEFAEGTRGIALNLESKNVGIVLMGDGLMIQEGSFVKATGRIAQIPVSEAYLGRVVNALAKPIDGRGEIIASESRLIESPAPSIISRRSVYEPLQTGLIAIDSMIPIGRGQRELIIGDRQTGKTAVATDTILNQKGQGVICVYVAIGQRASS
VAQVVTTFHEEGAMEYTIVVAEMADSPATLQYLAPYTGAALAEYFMYRERHTLIIYDDLSKQAQAYRQMSLLLRRPPGREAYPGDVFYLHSRLLERAAKLNSLLGEGSMTALPIVETQSGDVSAYIPTNVISITDGQIFLSADLFNAGIRPAINVGISVSRVGSAAQIKAMKQVAGKSKLELAQFAELQAFAQFASALDKTSQNQ
LARGRRLRELLKQSQANPLPVEEQIATIYTGTRGYLDSLEIEQVNKFLDELRKHLKDTKPQFQEIISSSKTFTEQAEILLKEAIQEQLERFSLQ

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Protein spot 11

Protein spot 12

Protein spot 13

Protein spot 14

ProteinPilot™ Software - [Result - E:\Adrian LCMS data\Adrian_3302.group]

File Configure Window Help

Workflow Tasks: Identify Proteins; LC...; Spot-Based (MS only)...; Spot-Based (MS and MS/MS)...; View; Analysis Log...; Result...; Export; Peptide Summary...; Distinct Peptide Summary...; Protein Summary...; Spectrum Summary...; MGF Peaklist(s)...; mzIdentML...; Features...

Protein ID | Features | Spectra | Summary Statistics

Proteins Detected

N	Unused	Total	% Cov (95)	Accession #	Name	Species	Peptides(95%)	Gene Ontology	Pathway	Int	Function
2	31.98	31.98	39.6	sp|P12782|PGKH_WHEAT	Phosphoglycerate kinase, chloroplastic OS=Triticum aestivum PE=2 SV=1	WHEAT	19	chloroplast; ATP bin...	Carboh...		
4	25.98	25.98	37.9	sp|P26302|KPPR_WHEAT	Phosphoribulokinase, chloroplastic OS=Triticum aestivum PE=2 SV=1	WHEAT	18	chloroplast; ATP bin...	Carboh...		
5	12.13	12.13	16.5	sp|P46285|S17P_WHEAT	Sedoheptulose-1,7-bisphosphatase, chloroplastic OS=Triticum aestivum PE=2 SV=1	WHEAT	7	chloroplast; fructose...	Carboh...		
6	11.14	11.25	12.1	sp|P20858|ATPB_WHEAT	ATP synthase subunit beta, chloroplastic OS=Triticum aestivum GN=atpB PE=3 SV=1	WHEAT	5	chloroplast thylakoid...			Produces ATP from ADP in the presence of a...
9	8.00	8.00	9.2	sp|P27665|PSBO_WHEAT	Oxygen-evolving enhancer protein 1, chloroplastic OS=Triticum aestivum GN=PSBO PE=2 SV=1	WHEAT	4	chloroplast thylakoid...			Stabilizes the manganese cluster which is the...
10	6.87	6.97	23.4	sp|P26667|RBS2_WHEAT	Ribulose bisphosphate carboxylase small chain PW9, chloroplastic OS=Triticum aestivum PE=3 SV=1	WHEAT	4	chloroplast; monoox...			RuBisCO catalyzes two reactions: the carboxyl...
13	3.36	3.37	4.2	sp|P11383|RBL_WHEAT	Ribulose bisphosphate carboxylase large chain OS=Triticum aestivum GN=rbcL PE=1 SV=2	WHEAT	2	chloroplast; magnesi...			RuBisCO catalyzes two reactions: the carboxyl...
15	2.00	6.00	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	3.1	sp|P12463|PSBA_WHEAT	Photosystem II protein D1 OS=Triticum aestivum GN=psbA PE=3 SV=2	WHEAT	1	chloroplast thylakoid...			Photosystem II (PSII) is a light-driven water pl...
17	1.80	5.89	20.4	sp|P07398|RBS3_WHEAT	Ribulose bisphosphate carboxylase small chain clone 512 (Fragment) OS=Triticum aestivum PE=2 SV=1	WHEAT	3	chloroplast; monoox...			RuBisCO catalyzes two reactions: the carboxyl...
8	8.05	8.05	17.9	cont|000143	pdb|1FNI_A| A Chain A, Crystal Structure Of Porcine Beta Trypsin With 0.01% Polydocanol [Sus scrofa (contaminant)]	Sus scrofa (contaminant)	9				
11	4.00	4.00	7.8	tr|A0A0C4ERA6|A0A0C4ERA6...	Uncharacterized protein OS=Puccinia triticina (isolate 1-1 / race 1 (BBBD)) GN=PTTG_03323 PE=3 SV=1	PUCT1	2				
14	3.15	3.19	1.3	tr|A0A0C4FAC4|A0A0C4FAC4...	Uncharacterized protein OS=Puccinia triticina (isolate 1-1 / race 1 (BBBD)) GN=PTTG_10165 PE=4 SV=1	PUCT1	2				
18	1.60	1.62	1.4	tr|A0A0C4EXC6|A0A0C4EXC6...	Uncharacterized protein OS=Puccinia triticina (isolate 1-1 / race 1 (BBBD)) GN=PTTG_05474 PE=4 SV=1	PUCT1	1				
1	49.32	49.32	33.5	cont|000135	cra|hCP1609934.2| keratin 1 (epidermolytic hyperkeratosis) [Homo sapiens (contaminant)]	Homo sapiens (contaminant)	26				
3	30.91	31.13	21.8	cont|000136	cra|hCP1812061| keratin 10 (epidermolytic hyperkeratosis; keratosis palmaris et plantaris) [Homo sapiens (contaminant)]	Homo sapiens (contaminant)	19				
7	9.44	12.18	10.3	cont|000126	spt|P48666| Keratin, type II cytoskeletal 6C (Cytokeratin 6C) (CK 6C) (K6c keratin) [Homo sapiens (contaminant)]	Homo sapiens (contaminant)	6				
12	3.57	3.59	12.7	cont|000124	spt|P81605| Dermcidin precursor (Preproteolysin) (Contains: Survival-promoting peptide; DCD-1) [Homo sapiens (cont...	Homo sapiens (contaminant)	2				

Protein Group 4 - Phosphoribulokinase, chloroplastic OS=Triticum aestivum PE=2 SV=1 ☑ Remove Redundancy

Proteins in Group

N	Unused	Total	Accessio...	Name	Species
4	25.98	25.98	sp|P2630...	Phosphoribulokina...	WHEAT

Peptides in Group

Peptide Locus	Con...	Conf	Sequence	Modifications	Protein Modifications	Cleavages	ΔMass	Obs MW	z	Sc	Spectrum	Type
114.001	2.00	97.1	AIEKPIYNHVTGLLDPAELIQPPK				0.0049	2655.46...	4	9	1.1.1.144.5	Winner
105.001	2.00	99	AVEQPIVIGLAADSGCGK	Carbamidomethyl(C)@16		cleaved C-...	0.0040	1783.91...	3	18	1.1.1.127.7	Winner
141.001	2.00	99	DLYEQIIAER				-0.0001	1248.63...	2	13	1.1.1.147.5	Winner
130.001	2.00	99	FYGEVTQQMLK	Oxidation(M)@9			-0.0005	1358.65...	2	11	1.1.1.87.16	Winner
139.001	2.00	99	IRDLYEQIIAER			missed R-D...	0.0049	1517.82...	3	14	1.1.1.124.3	Winner
126.001	2.00	99	KLTCSYPGIK	Carbamidomethyl(C)@4		missed K-L...	-0.0026	1165.61...	2	9	1.1.1.58.8	Winner
123.001	2.00	99	KPDFDAFIDPQK				0.0029	1419.70...	3	10	1.1.1.107.3	Winner
128.001	2.00	99	LDELIYVESHLSNLSTK				-0.0038	1960.01...	3	12	1.1.1.156.6	Winner
108.001	2.00	99	LTSVFGGAAEPPK				0.0012	1272.67...	2	12	1.1.1.93.15	Winner
117.001	2.00	99	PAELIQPPK			cleaved D-...	-0.0015	991.5688	2	14	1.1.1.74.7	Winner
107.001	2.00	99	RLTSVFGGAAEPPK			missed R-L...	-0.0035	1428.76...	3	16	1.1.1.77.7	Winner
131.001	1.55	98.6	HADFPGSNNGTGLFQTIVGLK				-0.0010	2172.09...	3	8	1.1.1.155.4	Winner
118.001	1.11	99	IFVIEGLHPMYDER	Oxidation(P)@9 …			-0.0019	1749.83...	3	9	1.1.1.129.7	Winner

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

MAFCSPHTTTSLRSPCTTIPNSGFRQNQVIFFTTRSSRRSNTRRGARTFQVSCAVEQPIVIGLAADSGCGKSTFMRRLTSVFGGAAEPPKGGNPDSNTLISDTTTVICLDDYHSLDRTGRKENGVTALDPRANDFDLMYEQVKAIKEGKAIEKPIYNHVTGLLDPAELIQPPKIFVIEGLHPMYDERVRELLDFSIYLDISNEVK
FAWKIQRDMAERGHSLESIKASIEARKPDFDAFIDPQKQYADAVIEVLPTQLIPDDNEGKVLRVKLIMKEGIKFFNPVYLFDEGSTINKIPCGRKLTCSYPGIKFSYGPDTYFGQEVSVLEMDGQFDRLDELIYVESHLSNLSTKFYGEVTQQMLKHADFPGSNNGTGLFQTIVGLKIRDLYEQIIAERAGVPAEAAKV

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ProteinPilot™ Software - [Result - E:\Adrian LCMS data\Adrian_3302.group]

File Configure Window Help

Workflow Tasks: Identify Proteins; LC...; Spot-Based (MS only)...; Spot-Based (MS and MS/MS)...; View; Analysis Log...; Result...; Export; Peptide Summary...; Distinct Peptide Summary...; Protein Summary...; Spectrum Summary...; MGF Peaklist(s)...; mzIdentML...; Features...

Protein ID | Features | Spectra | Summary Statistics

Proteins Detected

N	Unused	Total	% Cov (95)	Accession #	Name	Species	Peptides(95%)	Gene Ontology	Pathway	Int	Function
2	31.98	31.98	39.6	sp|P12782|PGKH_WHEAT	Phosphoglycerate kinase, chloroplastic OS=Triticum aestivum PE=2 SV=1	WHEAT	19	chloroplast; ATP bin...	Carboh...		
4	25.98	25.98	37.9	sp|P26302|KPPR_WHEAT	Phosphoribulokinase, chloroplastic OS=Triticum aestivum PE=2 SV=1	WHEAT	18	chloroplast; ATP bin...	Carboh...		
5	12.13	12.13	16.5	sp|P46285|S17P_WHEAT	Sedoheptulose-1,7-bisphosphatase, chloroplastic OS=Triticum aestivum PE=2 SV=1	WHEAT	7	chloroplast; fructose...	Carboh...		
6	11.14	11.25	12.1	sp|P20858|ATPB_WHEAT	ATP synthase subunit beta, chloroplastic OS=Triticum aestivum GN=atpB PE=3 SV=1	WHEAT	5	chloroplast thylakoid...			Produces ATP from ADP in the presence of a...
9	8.00	8.00	9.2	sp|P27665|PSBO_WHEAT	Oxygen-evolving enhancer protein 1, chloroplastic OS=Triticum aestivum GN=PSBO PE=2 SV=1	WHEAT	4	chloroplast thylakoid...			Stabilizes the manganese cluster which is the...
10	6.87	6.97	23.4	sp|P26667|RBS2_WHEAT	Ribulose bisphosphate carboxylase small chain PW9, chloroplastic OS=Triticum aestivum PE=3 SV=1	WHEAT	4	chloroplast; monoox...			RuBisCO catalyzes two reactions: the carboxyl...
13	3.36	3.37	4.2	sp|P11383|RBL_WHEAT	Ribulose bisphosphate carboxylase large chain OS=Triticum aestivum GN=rbcL PE=1 SV=2	WHEAT	2	chloroplast; magnesi...			RuBisCO catalyzes two reactions: the carboxyl...
15	2.00	6.00	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	3.1	sp|P12463|PSBA_WHEAT	Photosystem II protein D1 OS=Triticum aestivum GN=psbA PE=3 SV=2	WHEAT	1	chloroplast thylakoid...			Photosystem II (PSII) is a light-driven water pl...
17	1.80	5.89	20.4	sp|P07398|RBS3_WHEAT	Ribulose bisphosphate carboxylase small chain clone 512 (Fragment) OS=Triticum aestivum PE=2 SV=1	WHEAT	3	chloroplast; monoox...			RuBisCO catalyzes two reactions: the carboxyl...
8	8.05	8.05	17.9	cont|000143	pdb|1FNI_A| A Chain A, Crystal Structure Of Porcine Beta Trypsin With 0.01% Polydocanol [Sus scrofa (contaminant)]	Sus scrofa (contaminant)	9				
11	4.00	4.00	7.8	tr|A0A0C4ERA6|A0A0C4ERA6...	Uncharacterized protein OS=Puccinia triticina (isolate 1-1 / race 1 (BBBD)) GN=PTTG_03323 PE=3 SV=1	PUCT1	2				
14	3.15	3.19	1.3	tr|A0A0C4FAC4|A0A0C4FAC4...	Uncharacterized protein OS=Puccinia triticina (isolate 1-1 / race 1 (BBBD)) GN=PTTG_10165 PE=4 SV=1	PUCT1	2				
18	1.60	1.62	1.4	tr|A0A0C4EXC6|A0A0C4EXC6...	Uncharacterized protein OS=Puccinia triticina (isolate 1-1 / race 1 (BBBD)) GN=PTTG_05474 PE=4 SV=1	PUCT1	1				
1	49.32	49.32	33.5	cont|000135	cra|hCP1609934.2| keratin 1 (epidermolytic hyperkeratosis) [Homo sapiens (contaminant)]	Homo sapiens (contaminant)	26				
3	30.91	31.13	21.8	cont|000136	cra|hCP1812061| keratin 10 (epidermolytic hyperkeratosis; keratosis palmaris et plantaris) [Homo sapiens (contaminant)]	Homo sapiens (contaminant)	19				
7	9.44	12.18	10.3	cont|000126	spt|P48666| Keratin, type II cytoskeletal 6C (Cytokeratin 6C) (CK 6C) (K6c keratin) [Homo sapiens (contaminant)]	Homo sapiens (contaminant)	6				
12	3.57	3.59	12.7	cont|000124	spt|P81605| Dermcidin precursor (Preproteolysin) (Contains: Survival-promoting peptide; DCD-1) [Homo sapiens (cont...	Homo sapiens (contaminant)	2				

Protein Group 5 - Sedoheptulose-1,7-bisphosphatase, chloroplastic OS=Triticum aestivum PE=2 SV=1 ☑ Remove Redundancy

Proteins in Group

N	Unused	Total	Accessio...	Name	Species
5	12.13	12.13	sp|P4628...	Sedoheptulose-1,7-b...	WHEAT

Peptides in Group

Peptide Locus	Con...	Conf	Sequence	Modifications	Protein Modifications	Cleavages	ΔMass	Obs MW	z	Sc	Spectrum	Type
155.001	2.00	99	FEETLYGSSR				0.0003	1187.54...	2	7	1.1.1.86.13	Winner
152.001	2.00	99	GIFTNVTSPTAK				-0.0020	1234.65...	2	9	1.1.1.93.14	Winner
150.001	2.00	99	LVNYYVK				0.0017	897.4977	2	7	1.1.1.79.5	Winner
148.001	2.00	99	MFSPGNLR	Oxidation(M)@1			-0.0019	936.4469	2	8	1.1.1.71.11	Winner
147.001	2.00	99	TTFVVALK				-0.0016	877.5257	2	10	1.1.1.103.3	Winner
154.001	2.00	99	VISVLDER				-0.0020	929.5163	2	10	1.1.1.87.4	Winner
149.001	0.12	40.3	ATFDNPDYDK				-0.0051	1184.49...	2	6	1.1.1.67.11	Winner
156.001	0.01	4.5	LAASATVGATA				-0.0032	931.4943	2	5	1.1.1.72.8	Winner
146.001	0.00	0.9	LTGVTGGDQVAAAMGIYGPR	Oxidation(M)@14			0.0024	1948.96...	3	4	1.1.1.114.6	Winner
143.001	0.00	96.2	AIGDSLEEFLTK			cleaved C-...	-0.0010	1321.67...	2	7	1.1.1.162.4	Winner
145.001	0.00	0.4	LLICMGEAMR	Carbamidomethyl(C)@4 Oxidation(M)@5 Oxidation(M)@9			-0.0032	1224.56...	2	3	1.1.1.75.11	Winner
152.002	0.00	0.2	IFTNVTSPTAK	Carbamidomethyl@N-term …		cleaved G-I...	-0.0020	1234.65...	2	8	1.1.1.93.14	Winner

Protein Sequence Coverage - Sedoheptulose-1,7-bisphosphatase, chloroplastic OS=Triticum aestivum PE=2 SV=1

METVAAAGYAHGAATRSPACCAAMSFSQSYRPKAARPATSFYGESLRANTARTSFPAGRQSKAASRAALTTRCAIGDSLEEFLTKATPDKNLIRLLICMGEAMRTIAFKVRTASCGGTACVNSFGDEQLAVDMLADKLLFEALEYSHVCKYACSEEVPELQDMGGPVEGGFSVAFDPLDGSSIVDTNFTVGTIFGVWPGDKLTGV
TGGDQVAAAMGIYGPRTTFVVALKDCPGTHEFLLLDEGKWQHVKDTTSIGEGKMFSPGNLRATFDNPDYDKLVNYYVKEKYTLRYTGGMVPDVNQIIVKEKGIFTNVTSPTAKAKLRLLFEVAPLGFLIEKAGGHSSDGKQSVLDKVISVLDERTQVAYGSKNEIIRFEETLYGSSRLAASATVGATA

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Protein spot 15

ProteinPilot™ Software - [Result - E:\Adrian LCMS data\Adrian_3306.group]

File Configure Window Help

Workflow Tasks: Identify Proteins; LC...; Spot-Based (MS only)...; Spot-Based (MS and MS/MS)...; View; Analysis Log...; Result...; Export; Peptide Summary...; Distinct Peptide Summary...; Protein Summary...; Spectrum Summary...; MGF Peaklist(s)...; mzIdentML...; Features...

Protein ID | Features | Spectra | Summary Statistics

Proteins Detected

N	Unused	Total	% Cov (95)	Accession #	Name	Species	Peptides(95%)	Gene Ontology	Pathway	Int	Function
3	13.37	13.37	30.5	sp|P27665|PSBO_WHEAT	Oxygen-evolving enhancer protein 1, chloroplastic OS=Triticum aestivum GN=PSBO PE=2 SV=1	WHEAT	14	chloroplast thylakoid...			Stabilizes the manganese cluster which is the...
5	4.01	4.01	8.6	sp|P00871|RBS1_WHEAT	Ribulose bisphosphate carboxylase small chain PWS4.3, chloroplastic OS=Triticum aestivum PE=3 SV=2	WHEAT	2	chloroplast, monoox...			RuBisCO catalyzes two reactions: the carboxyl...
6	2.00	4.01	13.3	sp|P07398|RBS3_WHEAT	Ribulose bisphosphate carboxylase small chain clone 512 (Fragment) OS=Triticum aestivum PE=2 SV=1	WHEAT	2	chloroplast, monoox...			RuBisCO catalyzes two reactions: the carboxyl...
8	1.45	1.47	1.8	sp|P20858|ATPB_WHEAT	ATP synthase subunit beta, chloroplastic OS=Triticum aestivum GN=atpB PE=3 SV=1	WHEAT	1	chloroplast thylakoid...			Produces ATP from ADP in the presence of a...
10	1.37	1.39	1.9	sp|P11383|RBL_WHEAT	Ribulose bisphosphate carboxylase large chain OS=Triticum aestivum GN=rbcL PE=1 SV=2	WHEAT	1	chloroplast, magnesi...			RuBisCO catalyzes two reactions: the carboxyl...
4	6.00	6.00	17.0	cont|000143	pdb|1FNI_A| A Chain A, Crystal Structure Of Porcine Beta Trypsin With 0.01% Polydocanol [Sus scrofa (contaminant)]	Sus scrofa (contaminant)	7				
7	2.00	2.00	1.9	tr|A0A0C4ELH7|A0A0C4ELH7...	Uncharacterized protein OS=Puccinia triticina (isolate 1-1 / race 1 (BBBD)) GN=PTTG_01611 PE=4 SV=1	PUCT1	1				
9	1.40	1.42	2.6	tr|A0A0C4EYA2|A0A0C4EYA2...	Uncharacterized protein OS=Puccinia triticina (isolate 1-1 / race 1 (BBBD)) GN=PTTG_05801 PE=3 SV=1	PUCT1	1				
1	17.62	17.62	14.0	cont|000133	pir|KRHU0| keratin 10, type I, cytoskeletal - human [Homo sapiens (contaminant)]	Homo sapiens (contaminant)	8				
2	16.91	16.91	11.8	cont|000136	cra|hCP1609934.2| keratin 1 (epidermolytic hyperkeratosis) [Homo sapiens (contaminant)]	Homo sapiens (contaminant)	8				

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	13.37	13.37	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
43.001	2.00	99	FCLEPTSFTVK	Carbamidomethyl(C)@2			0.0040	1327.65...	2	12	1.1.1.136.5	Winner
65.001	2.00	99	GDEEELAKENVK			missed K-E...	0.0089	1359.66...	3	16	1.1.1.52.3	Winner
59.001	2.00	99	GSSFLDPK				0.0070	849.4303	2	12	1.1.1.72.4	Winner
42.001	2.00	99	KFCLEPTSFTVK	Carbamidomethyl(C)@3		missed K-F...	0.0048	1455.74...	3	8	1.1.1.112.4	Winner
51.001	2.00	99	QLVATGKPESF			cleaved F-...	0.0038	1175.62...	2	11	1.1.1.85.2	Winner
34.001	2.00	99	RLTFDEIQSK			missed R-L...	0.0073	1235.65...	2	13	1.1.1.77.6	Winner
66.001	0.81	89.2	NASSSTGNITLSVTK				0.0054	1478.76...	2	6	1.1.1.81.5	Winner
35.001	0.48	99	LTFDEIQSK	Cation:Na(E)@5			-0.0012	1101.53...	2	12	1.1.1.97.3	Winner
50.001	0.06	99	VAFLFTVK	Delta:H(2)C(2)@N-term			0.0057	949.5695	2	11	1.1.1.147.3	Winner
68.001	0.02	99	IQGVWYAQ			cleaved Q-...	0.0031	963.4845	2	10	1.1.1.134.3	Winner
67.001	0.00	0.6	DVKIQGVWYAQLESN	Deamidated(N)@15		missed K-L...	-0.1136	1749.74...	3	3	1.1.1.364.5	Winner
47.001	0.00	0.1	LTYTLDEMEGPLEVR				0.0880	1764.94...	3	3	1.1.1.183.2	Winner
48.001	0.00	4.2	DGIDYAAVTVQLPGGER	Cation:Na(D)@1			0.0046	1781.86...	3	8	1.1.1.151.2	Winner
49.001	0.00	4.2	DGIDYAAVTVQLPGGER	Cation:Na(D)@4			0.0046	1781.86...	3	8	1.1.1.151.2	Winner
45.001	0.00	1.2	FCLEPTSFTVK	Carbamidomethyl(C)@2 Cation:K(E)@4			-0.0002	1365.60...	3	6	1.1.1.136.2	Winner
44.001	0.00	3.7	FCLEPTSFTVK	Lys-add@N-term Carbamidomethyl(C)@2			0.0048	1455.74...	3	8	1.1.1.112.4	Winner
64.001	0.00	0.2	GGSTGYDNAGALPRGGR	Arg->GluSA(R)@14		missed R-...	0.0396	1561.75...	2	5	1.1.1.89.9	Winner
63.001	0.00	0.2	GGSTGYDNAGALPRGGR	Arg->GluSA(R)@17		missed R-...	0.0396	1561.75...	2	5	1.1.1.89.9	Winner

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

MAASLQAAATVMPAKIGGRASSARPSSHVARAFGVDAGARITCSLQSDIREVASKCADAAKMAGFALATSALLVSGATAEGAPKRLTFDEIQSKTYMEVKGTGTANQCPTIDGGVDSFPFKAGKYEMKKFCLEPTSFTVKAEGIQKNEPPRFQKTKLMTRLTYTLDEMEGPLEVRRRRTLKFEEKDGIDYAAVTVQLPGGERVAF
LFTVKQLVATGKPESFRPFLVPSYRGSSFLDPKGGGSTGYDNAGALPRGGRGDEEELAKENVKNASSSTGNITLSVTKSKPETGEVIGVFESVQPSDTDLEAPKDVKIQGVWYAQLESN

09:53 PM 2016/01/17

ProteinPilot™ Software - [Result - E:\Adrian LCMS data\Adrian_3306.group]

File Configure Window Help

Workflow Tasks: Identify Proteins; LC...; Spot-Based (MS only)...; Spot-Based (MS and MS/MS)...; View; Analysis Log...; Result...; Export; Peptide Summary...; Distinct Peptide Summary...; Protein Summary...; Spectrum Summary...; MGF Peaklist(s)...; mzIdentML...; Features...

Protein ID | Features | Spectra | Summary Statistics

Proteins Detected

N	Unused	Total	% Cov (95)	Accession #	Name	Species	Peptides(95%)	Gene Ontology	Pathway	Int	Function
3	13.37	13.37	30.5	sp|P27665|PSBO_WHEAT	Oxygen-evolving enhancer protein 1, chloroplastic OS=Triticum aestivum GN=PSBO PE=2 SV=1	WHEAT	14	chloroplast thylakoid...			Stabilizes the manganese cluster which is the...
5	4.01	4.01	8.6	sp|P00871|RBS1_WHEAT	Ribulose bisphosphate carboxylase small chain PWS4.3, chloroplastic OS=Triticum aestivum PE=3 SV=2	WHEAT	2	chloroplast, monoox...			RuBisCO catalyzes two reactions: the carboxyl...
6	2.00	4.01	13.3	sp|P07398|RBS3_WHEAT	Ribulose bisphosphate carboxylase small chain clone 512 (Fragment) OS=Triticum aestivum PE=2 SV=1	WHEAT	2	chloroplast, monoox...			RuBisCO catalyzes two reactions: the carboxyl...
8	1.45	1.47	1.8	sp|P20858|ATPB_WHEAT	ATP synthase subunit beta, chloroplastic OS=Triticum aestivum GN=atpB PE=3 SV=1	WHEAT	1	chloroplast thylakoid...			Produces ATP from ADP in the presence of a...
10	1.37	1.39	1.9	sp|P11383|RBL_WHEAT	Ribulose bisphosphate carboxylase large chain OS=Triticum aestivum GN=rbcL PE=1 SV=2	WHEAT	1	chloroplast, magnesi...			RuBisCO catalyzes two reactions: the carboxyl...
4	6.00	6.00	17.0	cont|000143	pdb|1FNI_A| A Chain A, Crystal Structure Of Porcine Beta Trypsin With 0.01% Polydocanol [Sus scrofa (contaminant)]	Sus scrofa (contaminant)	7				
7	2.00	2.00	1.9	tr|A0A0C4ELH7|A0A0C4ELH7...	Uncharacterized protein OS=Puccinia triticina (isolate 1-1 / race 1 (BBBD)) GN=PTTG_01611 PE=4 SV=1	PUCT1	1				
9	1.40	1.42	2.6	tr|A0A0C4EYA2|A0A0C4EYA2...	Uncharacterized protein OS=Puccinia triticina (isolate 1-1 / race 1 (BBBD)) GN=PTTG_05801 PE=3 SV=1	PUCT1	1				
1	17.62	17.62	14.0	cont|000133	pir|KRHU0| keratin 10, type I, cytoskeletal - human [Homo sapiens (contaminant)]	Homo sapiens (contaminant)	8				
2	16.91	16.91	11.8	cont|000136	cra|hCP1609934.2| keratin 1 (epidermolytic hyperkeratosis) [Homo sapiens (contaminant)]	Homo sapiens (contaminant)	8				

Protein Group 5 - Ribulose bisphosphate carboxylase small chain PWS4.3, chloroplastic OS=Triticum aestivum PE=3 SV=2 ☑ Remove Redundancy

Proteins in Group

N	Unused	Total	Accessio...	Name	Species
5	4.01	4.01	sp|P0087...	Ribulose bisphosph...	WHEAT
6	2.00	4.01	sp|P0739...	Ribulose bisphosph...	WHEAT

Peptides in Group

Peptide Locus	Con...	Conf	Sequence	Modifications	Protein Modifications	Cleavages	ΔMass	Obs MW	z	Sc	Spectrum	Type
77.001	2.00	99	QVDYLIR				0.0040	905.6011	2	7	1.1.1.94.3	Winner
79.001	2.00	99	VIGFDNLR				0.0041	932.5121	2	8	1.1.1.110.2	Winner
78.001	0.01	4	EYPDAYVR				0.0019	1011.46...	2	6	1.1.1.81.3	Winner
76.001	0.00	0.2	FETLSYLPPLSTEALSK	Lys-add@N-term Delta:H(2)C(2)(K)@17			0.0401	2049.14...	3	7	1.1.1.168.4	Winner
98.002	0.00	5.7	KFETLSYLPPLSTEALSK	Delta:H(2)C(2)(K)@18		missed K-F...	0.0401	2049.14...	3	7	1.1.1.168.4	Winner
80.001	2.00	99	IIGFDNMR	Oxidation(M)@7			0.0063	980.4813	2	8	1.1.1.84.4	Other
81.001	0.00	0.1	IIGFDNMRQVQCVSFIAFKPPGCEES...	Oxidation(M)@7 Carbamidomethyl(C)@12 Carbamidomethyl(C)@23		missed R-Q...	0.1062	3300.67...	6	3	1.1.1.311.7	Other

Protein Sequence Coverage - Ribulose bisphosphate carboxylase small chain PWS4.3, chloroplastic OS=Triticum aestivum PE=3 SV=2

MAPAVMASSATTVAPFQGLKSTAGLPVSRRSRGSLGSVSNGGRIRCMQVWPIEGIKKFETLSYLPPLSTEALSKQVDYLIRSKWVPCLEFSKVGFVFREHNSSPGYYDGRYWTMWKLPMFGCTDATQVLNEVEEVKKEYPDAYVRVIGFDNLRQVQCVSFIAFRPPGCEESGKA

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Protein spot 16

Protein spot 17

Protein Group 14 - Ribulose bisphosphate carboxylase small chain clone 512 (Fragment) OS=Triticum aestivum PE=2 SV=1

Protein Sequence Coverage - Ribulose bisphosphate carboxylase small chain clone 512 (Fragment) OS=Triticum aestivum PE=2 SV=1

Protein Group 4 - 2-Cys peroxiredoxin BAS1, chloroplastic (Fragment) OS=Triticum aestivum GN=TSA PE=1 SV=2

Protein Sequence Coverage - 2-Cys peroxiredoxin BAS1, chloroplastic (Fragment) OS=Triticum aestivum GN=TSA PE=1 SV=2

Protein Group 4 - Thioredoxin M-type, chloroplastic OS=Triticum aestivum PE=2 SV=1

Protein Sequence Coverage - Thioredoxin M-type, chloroplastic OS=Triticum aestivum PE=2 SV=1

ProteinPilot™ Software - [Result - E:\Adrian LCMS data\Adrian_1302.group]

File Configure Window Help

Workflow Tasks: Identify Proteins; LC...; Spot-Based (MS only)...; Spot-Based (MS and MS/MS)...; View; Analysis Log...; Result...; Export; Peptide Summary...; Distinct Peptide Summary...; Protein Summary...; Spectrum Summary...; MGF Peaklist(s)...; mzIdentML...; Features...

Protein ID | Features | Spectra | Summary Statistics

Proteins Detected

N	Unused	Total	% Cov (...)	Accession #	Name	Species	Peptides(95%)	Gene Ontology	Pathway	Int	Function
2	26.40	26.40	47.3	sp|Q00434|PSBP_WHEAT	Oxygen-evolving enhancer protein 2, chloroplastic OS=Triticum aestivum GN=PSBP PE=2 SV=1	WHEAT	19	chloroplast thylakoid...			May be involved in the regulation of photosyste...
4	18.33	18.41	46.7	sp|P80602|BAS1_WHEAT	2-Cys peroxiredoxin BAS1, chloroplastic (Fragment) OS=Triticum aestivum GN=TSA PE=1 SV=2	WHEAT	11	chloroplast; peroxida...			May be an antioxidant enzyme particularly in th...
5	10.09	10.09	37.2	cont|000141	spt|P00761| Trypsin precursor (EC 3.4.21.4) [Sus scrofa (contaminant)]	Sus scrofa (contaminant)	22				
6	8.02	8.02	36.4	sp|Q43199|APT1_WHEAT	Adenine phosphoribosyltransferase 1 OS=Triticum aestivum GN=APT1 PE=2 SV=1	WHEAT	6	cytoplasm; adenine...	Purine...		Catalyzes a salvage reaction resulting in the fo...
7	7.29	7.32	31.0	sp|P07398|RBS3_WHEAT	Ribulose bisphosphate carboxylase small chain clone 512 (Fragment) OS=Triticum aestivum PE=2 SV=1	WHEAT	4	chloroplast; monoox...			RuBisCO catalyzes two reactions: the carboxyl...
1	30.53	30.53	20.8	cont|000135	cra|hCP1609934.2| keratin 1 (epidermolytic hyperkeratosis) [Homo sapiens (contaminant)]	Homo sapiens (contaminant)	16				
3	19.62	19.62	13.5	cont|000136	cra|hCP1812061| keratin 10 (epidermolytic hyperkeratosis; keratosis palmaris et plantaris) [Homo sapiens (contaminant)]	Homo sapiens (contaminant)	10				
14	2.00	5.20	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...
10	3.31	3.31	12.7	cont|000124	spt|P81605| Dermcidin precursor (Preproteolysin) (Contains: Survival-promoting peptide DCD-1) [Homo sapiens (cont...	Homo sapiens (contaminant)	2				
9	6.42	6.45	7.9	sp|P26302|KPPR_WHEAT	Phosphoribulokinase, chloroplastic OS=Triticum aestivum PE=2 SV=1	WHEAT	3	chloroplast; ATP bin...	Carboh...		
8	7.17	7.22	7.5	sp|P11383|RBL_WHEAT	Ribulose bisphosphate carboxylase large chain OS=Triticum aestivum GN=rbcL PE=1 SV=2	WHEAT	4	chloroplast; magnesi...			RuBisCO catalyzes two reactions: the carboxyl...
19	1.70	1.80	6.5	sp|Q43217|H2B3_WHEAT	Histone H2B.3 OS=Triticum aestivum PE=1 SV=3	WHEAT	1	nucleosome; nucleu...			Core component of nucleosome. Nucleosomes...
11	2.96	2.96	6.2	sp|P27665|PSBO_WHEAT	Oxygen-evolving enhancer protein 1, chloroplastic OS=Triticum aestivum GN=PSBO PE=2 SV=1	WHEAT	2	chloroplast thylakoid...			Stabilizes the manganese cluster which is the...
13	2.36	5.51	4.6	cont|000127	spt|P48668| Keratin, type II cytoskeletal 6E (Cytokeratin 6E) (CK 6E) (K6e keratin) [Homo sapiens (contaminant)]	Homo sapiens (contaminant)	3				
12	2.75	2.78	4.4	sp|P20858|ATPB_WHEAT	ATP synthase subunit beta, chloroplastic OS=Triticum aestivum GN=atpB PE=3 SV=1	WHEAT	2	chloroplast thylakoid...			Produces ATP from ADP in the presence of a...
17	2.00	2.00	4.3	sp|Q43209|PIMT_WHEAT	Protein-L-isoaspartate O-methyltransferase OS=Triticum aestivum GN=PCM PE=1 SV=1	WHEAT	1	cytoplasm; protein-L...			Catalyzes the methyl esterification of L-isoaspa...
16	2.00	2.00	3.2	tr|A0A0C4EHI4|A0A0C4EHI4_...	Uridylate kinase OS=Puccinia triticina (isolate 1-1 / race 1 (BBBD)) GN=PTTG_00200 PE=3 SV=1	PUCT1	1				
15	2.00	2.00	2.1	sp|P12782|PGKH_WHEAT	Phosphoglycerate kinase, chloroplastic OS=Triticum aestivum PE=2 SV=1	WHEAT	1	chloroplast; ATP bin...	Carboh...		
18	1.96	2.00	2.0	sp|P46285|S17P_WHEAT	Sedoheptulose-1,7-bisphosphatase, chloroplastic OS=Triticum aestivum PE=2 SV=1	WHEAT	1	chloroplast; fructose...	Carboh...		
20	1.62	1.72	1.1	tr|A0A0C4FAC4|A0A0C4FAC4...	Uncharacterized protein OS=Puccinia triticina (isolate 1-1 / race 1 (BBBD)) GN=PTTG_10165 PE=4 SV=1	PUCT1	1				

Protein Group 17 - Protein-L-isoaspartate O-methyltransferase OS=Triticum aestivum GN=PCM PE=1 SV=1

Remove Redundancy

Proteins in Group

N	Unused	Total	Accessio...	Name	Species
17	2.00	2.00	sp|Q432...	Protein-L-isoaspartat...	WHEAT

Peptides in Group

Peptide Locus	Con...	Conf	Sequence	Modifications	Protein Modifications	Cleavages	ΔMass	Obs MW	z	Sc	Spectrum	Type
173.001	2.00	99	VAEVMETIDR	Oxidation(M)@5			-0.0017	1177.56...	2	9	1.1.1.54.13	Winner

Protein Sequence Coverage - Protein-L-isoaspartate O-methyltransferase OS=Triticum aestivum GN=PCM PE=1 SV=1

Protein spot 18

ProteinPilot™ Software - [Result - E:\Adrian LCMS data\Adrian_2401.group]

File Configure Window Help

Workflow Tasks: Identify Proteins; LC...; Spot-Based (MS only)...; Spot-Based (MS and MS/MS)...; View; Analysis Log...; Result...; Export; Peptide Summary...; Distinct Peptide Summary...; Protein Summary...; Spectrum Summary...; MGF Peaklist(s)...; mzIdentML...; Features...

Protein ID | Features | Spectra | Summary Statistics

Proteins Detected

N	Unused	Total	% Cov (95)	Accession #	Name	Species	Peptides(95%)	Gene Ontology	Pathway	Int	Function
5	6.05	6.05	16.2	sp|P04784|CB21_WHEAT	Chlorophyll a-b binding protein, chloroplastic OS=Triticum aestivum GN=WHAB1.6 PE=3 SV=1	WHEAT	4	chloroplast thylakoid...			The light-harvesting complex (LHC) functions a...
7	2.00	2.00	4.6	sp|P00871|RBS1_WHEAT	Ribulose bisphosphate carboxylase small chain PWS4.3, chloroplastic OS=Triticum aestivum PE=3 SV=2	WHEAT	1	chloroplast; monoox...			RuBisCO catalyzes two reactions: the carboxyl...
8	2.00	2.00	1.9	sp|P11383|RBL_WHEAT	Ribulose bisphosphate carboxylase large chain OS=Triticum aestivum GN=rbcL PE=1 SV=2	WHEAT	1	chloroplast; magnesi...			RuBisCO catalyzes two reactions: the carboxyl...
3	16.27	16.27	45.3	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				
9	1.60	1.64	1.8	tr|A0A0C4F128|A0A0C4F128_...	Uncharacterized protein OS=Puccinia triticina (isolate 1-1 / race 1 (BBBD)) GN=PTTG_06798 PE=3 SV=1	PUCT1	1				
10	1.48	1.52	1.9	tr|A0A0C4EWJ7|A0A0C4EWJ7...	Uncharacterized protein OS=Puccinia triticina (isolate 1-1 / race 1 (BBBD)) GN=PTTG_05192 PE=4 SV=1	PUCT1	1				
1	53.71	53.71	32.0	cont|000135	cra|hCP1609934.2| keratin 1 (epidermolytic hyperkeratosis) [Homo sapiens (contaminant)]	Homo sapiens (contaminant)	34				
2	41.19	41.19	32.4	cont|000136	cra|hCP1812061| keratin 10 (epidermolytic hyperkeratosis; keratosis palmaris et plantaris) [Homo sapiens (contaminant)]	Homo sapiens (contaminant)	33				
4	14.79	19.71	19.4	cont|000126	spt|P48666| Keratin, type II cytoskeletal 6C (Cytokeratin 6C) (CK 6C) (K6c keratin) [Homo sapiens (contaminant)]	Homo sapiens (contaminant)	12				
6	4.03	4.03	12.7	cont|000124	spt|P81605| Dermcidin precursor (Preproteolysin) (Contains: Survival-promoting peptide DCD-1) [Homo sapiens (cont...	Homo sapiens (contaminant)	3				

Protein Group 5 - Chlorophyll a-b binding protein, chloroplastic OS=Triticum aestivum GN=WHAB1.6 PE=3 SV=1

Remove Redundancy

Proteins in Group

N	Unused	Total	Accessio...	Name	Species
5	6.05	6.05	sp|P0478...	Chlorophyll a-b bindi...	WHEAT

Peptides in Group

Peptide Locus	Con...	Conf	Sequence	Modifications	Protein Modifications	Cleavages	ΔMass	Obs MW	z	Sc	Spectrum	Type
212.001	2.00	99	ATNFVPGK			cleaved I-A...	0.0004	832.4447	2	10	1.1.1.48.3	Winner
209.001	2.00	99	LAMFSMFGFFVQAIVTGK	Oxidation(M)@3 Oxidation(M)@6			-0.0023	2025.00...	3	10	1.1.1.219.3	Winner
208.001	2.00	99	PQAFAELK	Deamidated(Q)@2		cleaved R-...	-0.0007	903.4695	2	12	1.1.1.77.3	Winner
206.001	0.04	98.9	SADPETFAK			cleaved L-...	-0.0024	964.4479	2	6	1.1.1.50.2	Winner
207.001	0.01	82.8	ELEVIHCR	Carbamidomethyl(C)@7			-0.0344	1054.48...	2	6	1.1.1.52.5	Winner
210.001	0.00	7.9	LAMFSMFGFFVQAIVTGK	Dioxidation(M)@3 Oxidation(M)@6			-0.0030	2041.00...	3	6	1.1.1.214.5	Winner
211.001	0.00	1.2	LAMFSMFGFFVQAIVTGK	Oxidation(M)@3 Dioxidation(M)@6			-0.0030	2041.00...	3	5	1.1.1.214.5	Winner
212.002	0.00	0.1	TNFVPGK	Propionamide@N-term		cleaved A-...	0.0004	832.4447	2	8	1.1.1.48.3	Winner

Protein Sequence Coverage - Chlorophyll a-b binding protein, chloroplastic OS=Triticum aestivum GN=WHAB1.6 PE=3 SV=1

Protein spot 19

Proteins Detected

N	Unused	Total	% Cov (95)	Accession #	Name	Species	Peptides(95%)	Gene Ontology	Pathway	Int	Function
2	37.96	37.96	57.8	sp|Q00434|PSBP_WHEAT	Oxygen-evolving enhancer protein 2, chloroplastic OS=Triticum aestivum GN=PSBP PE=2 SV=1	WHEAT	35	chloroplast thylakoid...			May be involved in the regulation of photosy
5	11.54	11.54	12.5	sp|P12112|ATPA_WHEAT	ATP synthase subunit alpha, chloroplastic OS=Triticum aestivum GN=atpA PE=3 SV=2	WHEAT	6	chloroplast thylakoid...			Produces ATP from ADP in the presence of a
7	8.88	8.92	23.4	sp|P26667|RBS2_WHEAT	Ribulose bisphosphate carboxylase small chain PW9, chloroplastic OS=Triticum aestivum PE=3 SV=1	WHEAT	4	chloroplast; monoox...			RuBisCO catalyzes two reactions: the carbox
8	7.42	7.42	9.0	sp|P11383|RBL_WHEAT	Ribulose bisphosphate carboxylase large chain OS=Triticum aestivum GN=rbcL PE=1 SV=2	WHEAT	4	chloroplast; magnesi...			RuBisCO catalyzes two reactions: the carbox
10	4.71	4.73	12.8	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
11	4.00	4.00	4.0	sp|P12782|PGKH_WHEAT	Phosphoglycerate kinase, chloroplastic OS=Triticum aestivum PE=2 SV=1	WHEAT	2	chloroplast; ATP bin...	Carboh...		
12	2.87	2.88	2.2	sp|P20858|ATPB_WHEAT	ATP synthase subunit beta, chloroplastic OS=Triticum aestivum GN=atpB PE=3 SV=1	WHEAT	1	chloroplast thylakoid...			Produces ATP from ADP in the presence of a
13	2.44	2.46	4.3	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
15	2.01	3.67	3.3	sp|P12862|ATPAM_WHEAT	ATP synthase subunit alpha, mitochondrial OS=Triticum aestivum GN=ATPA PE=3 SV=1	WHEAT	2	mitochondrial inner...			Mitochondrial membrane ATP synthase (F(1)
16	2.00	6.84	20.4	sp|P07398|RBS3_WHEAT	Ribulose bisphosphate carboxylase small chain clone 512 (Fragment) OS=Triticum aestivum PE=2 SV=1	WHEAT	3	chloroplast; monoox...			RuBisCO catalyzes two reactions: the carbox
17	2.00	6.84	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 carbox
18	2.00	2.00	4.3	sp|Q43209|PIMT_WHEAT	Protein-L-isoaspartate O-methyltransferase OS=Triticum aestivum GN=PCM PE=1 SV=1	WHEAT	1	cytoplasm; protein-L-...			Catalyzes the methyl esterification of L-isoas
19	2.00	2.00	5.9	sp|P27807|H2B1_WHEAT	Histone H2B.1 OS=Triticum aestivum PE=1 SV=3	WHEAT	1	nucleosome; nucleu...			Core component of nucleosome. Nucleosom
4	20.09	20.09	34.1	cont|000040	gi|2914482|pdb|1TFX|A Chain A, Complex Of The Second Kunitz Domain Of Tissue Factor Pathway Inhibitor With Por...	Sus scrofa (contaminant)	23				
20	2.00	2.00	3.2	tr|A0A0C4EHI4|A0A0C4EHI4_...	Uridylate kinase OS=Puccinia triticina (isolate 1-1 / race 1 (BBBD)) GN=PTTG_00200 PE=3 SV=1	PUCT1	1				
21	1.77	1.89	0.5	tr|A0A0C4F2R2|A0A0C4F2R2...	Uncharacterized protein OS=Puccinia triticina (isolate 1-1 / race 1 (BBBD)) GN=PTTG_07383 PE=4 SV=1	PUCT1	1				
1	43.01	43.01	27.6	cont|000135	cra|hCP1609934.2| keratin 1 (epidermolytic hyperkeratosis) [Homo sapiens (contaminant)]	Homo sapiens (contaminant)	24				
3	34.44	34.44	21.6	cont|000136	cra|hCP1812061| keratin 10 (epidermolytic hyperkeratosis; keratosis palmaris et plantaris) [Homo sapiens (contaminant)]	Homo sapiens (contaminant)	17				
6	9.31	11.56	10.3	cont|000126	spt|P48666| Keratin, type II cytoskeletal 6C (Cytokeratin 6C) (CK 6C) (K6c keratin) [Homo sapiens (contaminant)]	Homo sapiens (contaminant)	6				
14	2.42	2.44	12.7	cont|000124	spt|P81605| Dermcidin precursor (Preproteolysin) (Contains: Survival-promoting peptide DCD-1) [Homo sapiens (cont...	Homo sapiens (contaminant)	1				
9	5.96	5.96	4.9	cont|000097	gi|1351907|sp|P02769|ALBU_BOVIN Serum albumin precursor (Allergen Bos d 6) (BSA) [Bos taurus (contaminant)]	Bos taurus (contaminant)	3				

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

Proteins in Group

N	Unused	Total	Accessio...	Name	Species
2	37.96	37.96	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
43.001	2.00	99	AYGEAANVFGK			cleaved A-...	0.0039	1125.54...	2	12	1.1.1.82.15	Winner
69.001	2.00	99	EFPGQVLR				0.0009	944.5089	2	13	1.1.1.88.9	Winner
68.001	2.00	99	EREFPGQVLR			missed R-E...	0.0021	1229.65...	3	12	1.1.1.67.4	Winner
100.001	2.00	99	FVENAAGSF			cleaved F-...	-0.0024	940.4267	2	12	1.1.1.107.3	Winner
88.001	2.00	99	HQLITATVADGK				0.0031	1252.68...	3	15	1.1.1.55.3	Winner
97.001	2.00	99	KFVENAAGSF			cleaved F-...	0.0001	1068.52...	2	11	1.1.1.80.10	Winner
96.001	2.00	99	KFVENAAGSFSVA			missed K-F...	0.0027	1325.66...	2	11	1.1.1.97.12	Winner
52.001	2.00	99	KNTDFVAY			cleaved Y-...	-0.0002	956.4603	2	9	1.1.1.80.4	Winner
51.001	2.00	99	KNTDFVAYSGEGFK			missed K-N...	0.0016	1561.74...	3	12	1.1.1.85.7	Winner
78.001	2.00	99	LLGQQSYGGK			cleaved F-L...	0.0007	1049.55...	2	12	1.1.1.55.10	Winner
60.001	2.00	99	LMIPAKWNPSK	Oxidation(M)@2 Oxidation(W)@7		missed K-...	-0.0007	1315.69...	3	17	1.1.1.71.3	Winner

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

Protein Group 10 - Chlorophyll a-b binding protein, chloroplastic OS=Triticum aestivum GN=WHAB1.6 PE=3 SV=1

Proteins in Group

N	Unused	Total	Accessio...	Name	Species
10	4.71	4.73	sp|P0478...	Chlorophyll a-b bindi...	WHEAT

Peptides in Group

Peptide Locus	Con...	Conf	Sequence	Modifications	Protein Modifications	Cleavages	ΔMass	Obs MW	z	Sc	Spectrum	Type
231.001	2.00	99	ATNFVPGK			cleaved I-A...	0.0023	832.4467	2	11	1.1.1.49.2	Winner
227.001	1.92	99	ELEVIHCR	Carbamidomethyl(C)@7			-0.0011	1054.52...	2	7	1.1.1.54.13	Winner
228.001	0.79	95.5	LAMFSMFGFFVQAIVTGK	Oxidation(M)@3 Oxidation(M)@6			-0.0030	2025.00...	3	6	1.1.1.219.4	Winner
229.001	0.00	0.8	LAMFSMFGFFVQAIVTGK	Dioxidation(M)@3 Oxidation(M)@6			0.0035	2041.00...	3	4	1.1.1.215.5	Winner
230.001	0.00	0.8	LAMFSMFGFFVQAIVTGK	Oxidation(M)@3 Dioxidation(M)@6			0.0035	2041.00...	3	4	1.1.1.215.5	Winner
231.002	0.00	0.1	TNFVPGK	Propionamide@N-term		cleaved A-...	0.0023	832.4467	2	9	1.1.1.49.2	Winner

Protein Sequence Coverage - Chlorophyll a-b binding protein, chloroplastic OS=Triticum aestivum GN=WHAB1.6 PE=3 SV=1

ProteinPilot™ Software - [Result - E:\Adrian LCMS data\Adrian_5401.group]

File Configure Window Help

Workflow Tasks: Identify Proteins; LC...; Spot-Based (MS only)...; Spot-Based (MS and MS/MS)...; View; Analysis Log...; Result...; Export; Peptide Summary...; Distinct Peptide Summary...; Protein Summary...; Spectrum Summary...; MGF Peaklist(s)...; mzIdentML...; Features...

Protein ID | Features | Spectra | Summary Statistics

Proteins Detected

N	Unused	Total	% Cov (95)	Accession #	Name	Species	Peptides(95%)	Gene Ontology	Pathway	Int	Function
2	37.96	37.96	57.8	sp|Q00434|PSBP_WHEAT	Oxygen-evolving enhancer protein 2, chloroplastic OS=Triticum aestivum GN=PSBP PE=2 SV=1	WHEAT	35	chloroplast thylakoid...			May be involved in the regulation of photosy
5	11.54	11.54	12.5	sp|P12112|ATPA_WHEAT	ATP synthase subunit alpha, chloroplastic OS=Triticum aestivum GN=atpA PE=3 SV=2	WHEAT	6	chloroplast thylakoid...			Produces ATP from ADP in the presence of a
7	8.88	8.92	23.4	sp|P26667|RBS2_WHEAT	Ribulose bisphosphate carboxylase small chain PW9, chloroplastic OS=Triticum aestivum PE=3 SV=1	WHEAT	4	chloroplast; monoox...			RuBisCO catalyzes two reactions: the carbox
8	7.42	7.42	9.0	sp|P11383|RBL_WHEAT	Ribulose bisphosphate carboxylase large chain OS=Triticum aestivum GN=rbcL PE=1 SV=2	WHEAT	4	chloroplast; magnesi...			RuBisCO catalyzes two reactions: the carbox
10	4.71	4.73	12.8	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
11	4.00	4.00	4.0	sp|P12782|PGKH_WHEAT	Phosphoglycerate kinase, chloroplastic OS=Triticum aestivum PE=2 SV=1	WHEAT	2	chloroplast; ATP bin...	Carboh...		
12	2.87	2.88	2.2	sp|P20858|ATPB_WHEAT	ATP synthase subunit beta, chloroplastic OS=Triticum aestivum GN=atpB PE=3 SV=1	WHEAT	1	chloroplast thylakoid...			Produces ATP from ADP in the presence of a
13	2.44	2.46	4.3	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
15	2.01	3.67	3.3	sp|P12862|ATPAM_WHEAT	ATP synthase subunit alpha, mitochondrial OS=Triticum aestivum GN=ATPA PE=3 SV=1	WHEAT	2	mitochondrial inner...			Mitochondrial membrane ATP synthase (F(1)
16	2.00	6.84	20.4	sp|P07398|RBS3_WHEAT	Ribulose bisphosphate carboxylase small chain clone 512 (Fragment) OS=Triticum aestivum PE=2 SV=1	WHEAT	3	chloroplast; monoox...			RuBisCO catalyzes two reactions: the carbox
17	2.00	6.84	13.2	sp|P00871|RBS1_WHEAT	Ribulose bisphosphate carboxylase small chain PW54.3, chloroplastic OS=Triticum aestivum PE=3 SV=2	WHEAT	3	chloroplast; monoox...			RuBisCO catalyzes two reactions: the carbox
18	2.00	2.00	4.3	sp|Q43209|PIMT_WHEAT	Protein-L-isoaspartate O-methyltransferase OS=Triticum aestivum GN=PCM PE=1 SV=1	WHEAT	1	cytoplasm; protein-L-...			Catalyzes the methyl esterification of L-isoas
19	2.00	2.00	5.9	sp|P27807|H2B1_WHEAT	Histone H2B.1 OS=Triticum aestivum PE=1 SV=3	WHEAT	1	nucleosome; nucleu...			Core component of nucleosome. Nucleosom
4	20.09	20.09	34.1	cont|000040	gi|2914482|pdb|1TFX|A Chain A, Complex Of The Second Kunitz Domain Of Tissue Factor Pathway Inhibitor With Por...	Sus scrofa (contaminant)	23				
20	2.00	2.00	3.2	tr|A0A0C4EHI4|A0A0C4EHI4_...	Uridylate kinase OS=Puccinia triticina (isolate 1-1 / race 1 (BBBD)) GN=PTTG_00200 PE=3 SV=1	PUCT1	1				
21	1.77	1.89	0.5	tr|A0A0C4F2R2|A0A0C4F2R2...	Uncharacterized protein OS=Puccinia triticina (isolate 1-1 / race 1 (BBBD)) GN=PTTG_07383 PE=4 SV=1	PUCT1	1				
1	43.01	43.01	27.5	cont|000135	cra|hCP1609934.2| keratin 1 (epidermolytic hyperkeratosis) [Homo sapiens (contaminant)]	Homo sapiens (contaminant)	24				
3	34.44	34.44	21.5	cont|000136	cra|hCP1812061| keratin 10 (epidermolytic hyperkeratosis; keratosis palmaris et plantaris) [Homo sapiens (contaminant)]	Homo sapiens (contaminant)	17				
6	9.31	11.56	10.3	cont|000126	spt|P48666| Keratin, type II cytoskeletal 6C (Cytokeratin 6C) (CK 6C) (K6c keratin) [Homo sapiens (contaminant)]	Homo sapiens (contaminant)	6				
14	2.42	2.44	12.7	cont|000124	spt|P81605| Dermcidin precursor (Preproteolysin) (Contains: Survival-promoting peptide; DCD-1) [Homo sapiens (cont...	Homo sapiens (contaminant)	1				
9	5.96	5.96	4.9	cont|000097	gi|1351907|sp|P02769|ALBU_BOVIN Serum albumin precursor (Allergen Bos d 6) (BSA) [Bos taurus (contaminant)]	Bos taurus (contaminant)	3				

Protein Group 13 - 2-Cys peroxiredoxin BAS1, chloroplastic (Fragment) OS=Triticum aestivum GN=TSA PE=1 SV=2

☑ Remove Redundancy

Proteins in Group

N	Unused	Total	Accessio...	Name	Species
13	2.44	2.46	sp|P8060...	2-Cys peroxiredoxin...	WHEAT

Peptides in Group

Peptide Locus	Con...	Conf	Sequence	Modifications	Protein Modifications	Cleavages	ΔMass	Obs MW	z	Sc	Spectrum	Type
239.001	2.00	99	YPLVSDVTK				-0.0042	1020.54...	2	10	1.1.1.86.9	Winner
240.001	0.44	91.5	GLFIIDK				0.0016	804.4761	2	8	1.1.1.113.2	Winner
241.001	0.00	1.1	EGVIQHSTINNLGIGR				-0.0016	1706.90...	3	3	1.1.1.88.12	Winner
240.003	0.00	0.2	LFIIDK	Carbamidomethyl@N-term		cleaved G-...	0.0016	804.4761	2	7	1.1.1.113.2	Winner

Protein Sequence Coverage - 2-Cys peroxiredoxin BAS1, chloroplastic (Fragment) OS=Triticum aestivum GN=TSA PE=1 SV=2

DARARSFVARAAAEYDLPLVGNKAPDFAAEAVFDQEFINVKLSDYIGKKYVILFFYPLDFTFVCPTEITAFSDRHEEFEKINTEILGVSVDSVFSHLAWVQTERKSGGLGDLKYPLVSDVTKSISKSFGVLIPDQGIALRGLFIIDKEGVIQHSTINNLGIGRSVDETLRTLRALQYVKKPDEVCPAGWKPGEKSMKPDPKGSKE
YFAAI

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ProteinPilot™ Software - [Result - E:\Adrian LCMS data\Adrian_5401.group]

File Configure Window Help

Workflow Tasks: Identify Proteins; LC...; Spot-Based (MS only)...; Spot-Based (MS and MS/MS)...; View; Analysis Log...; Result...; Export; Peptide Summary...; Distinct Peptide Summary...; Protein Summary...; Spectrum Summary...; MGF Peaklist(s)...; mzIdentML...; Features...

Protein ID | Features | Spectra | Summary Statistics

Proteins Detected

N	Unused	Total	% Cov (95)	Accession #	Name	Species	Peptides(95%)	Gene Ontology	Pathway	Int	Function
2	37.96	37.96	57.8	sp|Q00434|PSBP_WHEAT	Oxygen-evolving enhancer protein 2, chloroplastic OS=Triticum aestivum GN=PSBP PE=2 SV=1	WHEAT	35	chloroplast thylakoid...			May be involved in the regulation of photosy
5	11.54	11.54	12.5	sp|P12112|ATPA_WHEAT	ATP synthase subunit alpha, chloroplastic OS=Triticum aestivum GN=atpA PE=3 SV=2	WHEAT	6	chloroplast thylakoid...			Produces ATP from ADP in the presence of a
7	8.88	8.92	23.4	sp|P26667|RBS2_WHEAT	Ribulose bisphosphate carboxylase small chain PW9, chloroplastic OS=Triticum aestivum PE=3 SV=1	WHEAT	4	chloroplast; monoox...			RuBisCO catalyzes two reactions: the carbox
8	7.42	7.42	9.0	sp|P11383|RBL_WHEAT	Ribulose bisphosphate carboxylase large chain OS=Triticum aestivum GN=rbcL PE=1 SV=2	WHEAT	4	chloroplast; magnesi...			RuBisCO catalyzes two reactions: the carbox
10	4.71	4.73	12.8	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
11	4.00	4.00	4.0	sp|P12782|PGKH_WHEAT	Phosphoglycerate kinase, chloroplastic OS=Triticum aestivum PE=2 SV=1	WHEAT	2	chloroplast; ATP bin...	Carboh...		
12	2.87	2.88	2.2	sp|P20858|ATPB_WHEAT	ATP synthase subunit beta, chloroplastic OS=Triticum aestivum GN=atpB PE=3 SV=1	WHEAT	1	chloroplast thylakoid...			Produces ATP from ADP in the presence of a
13	2.44	2.46	4.3	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
15	2.01	3.67	3.3	sp|P12862|ATPAM_WHEAT	ATP synthase subunit alpha, mitochondrial OS=Triticum aestivum GN=ATPA PE=3 SV=1	WHEAT	2	mitochondrial inner...			Mitochondrial membrane ATP synthase (F(1)
16	2.00	6.84	20.4	sp|P07398|RBS3_WHEAT	Ribulose bisphosphate carboxylase small chain clone 512 (Fragment) OS=Triticum aestivum PE=2 SV=1	WHEAT	3	chloroplast; monoox...			RuBisCO catalyzes two reactions: the carbox
17	2.00	6.84	13.2	sp|P00871|RBS1_WHEAT	Ribulose bisphosphate carboxylase small chain PW54.3, chloroplastic OS=Triticum aestivum PE=3 SV=2	WHEAT	3	chloroplast; monoox...			RuBisCO catalyzes two reactions: the carbox
18	2.00	2.00	4.3	sp|Q43209|PIMT_WHEAT	Protein-L-isoaspartate O-methyltransferase OS=Triticum aestivum GN=PCM PE=1 SV=1	WHEAT	1	cytoplasm; protein-L-...			Catalyzes the methyl esterification of L-isoas
19	2.00	2.00	5.9	sp|P27807|H2B1_WHEAT	Histone H2B.1 OS=Triticum aestivum PE=1 SV=3	WHEAT	1	nucleosome; nucleu...			Core component of nucleosome. Nucleosom
4	20.09	20.09	34.1	cont|000040	gi|2914482|pdb|1TFX|A Chain A, Complex Of The Second Kunitz Domain Of Tissue Factor Pathway Inhibitor With Por...	Sus scrofa (contaminant)	23				
20	2.00	2.00	3.2	tr|A0A0C4EHI4|A0A0C4EHI4_...	Uridylate kinase OS=Puccinia triticina (isolate 1-1 / race 1 (BBBD)) GN=PTTG_00200 PE=3 SV=1	PUCT1	1				
21	1.77	1.89	0.5	tr|A0A0C4F2R2|A0A0C4F2R2...	Uncharacterized protein OS=Puccinia triticina (isolate 1-1 / race 1 (BBBD)) GN=PTTG_07383 PE=4 SV=1	PUCT1	1				
1	43.01	43.01	27.5	cont|000135	cra|hCP1609934.2| keratin 1 (epidermolytic hyperkeratosis) [Homo sapiens (contaminant)]	Homo sapiens (contaminant)	24				
3	34.44	34.44	21.5	cont|000136	cra|hCP1812061| keratin 10 (epidermolytic hyperkeratosis; keratosis palmaris et plantaris) [Homo sapiens (contaminant)]	Homo sapiens (contaminant)	17				
6	9.31	11.56	10.3	cont|000126	spt|P48666| Keratin, type II cytoskeletal 6C (Cytokeratin 6C) (CK 6C) (K6c keratin) [Homo sapiens (contaminant)]	Homo sapiens (contaminant)	6				
14	2.42	2.44	12.7	cont|000124	spt|P81605| Dermcidin precursor (Preproteolysin) (Contains: Survival-promoting peptide; DCD-1) [Homo sapiens (cont...	Homo sapiens (contaminant)	1				
9	5.96	5.96	4.9	cont|000097	gi|1351907|sp|P02769|ALBU_BOVIN Serum albumin precursor (Allergen Bos d 6) (BSA) [Bos taurus (contaminant)]	Bos taurus (contaminant)	3				

Protein Group 18 - Protein-L-isoaspartate O-methyltransferase OS=Triticum aestivum GN=PCM PE=1 SV=1

☑ Remove Redundancy

Proteins in Group

N	Unused	Total	Accessio...	Name	Species
18	2.00	2.00	sp|Q432...	Protein-L-isoaspartat...	WHEAT

Peptides in Group

Peptide Locus	Con...	Conf	Sequence	Modifications	Protein Modifications	Cleavages	ΔMass	Obs MW	z	Sc	Spectrum	Type
252.001	2.00	99	VAEVMETIDR	Oxidation(M)@5			0.0006	1177.56...	2	9	1.1.1.59.11	Winner
253.001	0.00	0.2	ATCLELLK	Carbamidomethyl(C)@3		cleaved H-...	-0.0036	946.5121	2	4	1.1.1.92.3	Winner
254.001	0.00	0.7	ELVVASTENVER			cleaved P-...	0.0144	1344.70...	2	3	1.1.1.101.8	Winner
251.001	0.00	0.2	NNALVEYLK	Carbamidomethyl@N-term			0.0027	1119.59...	2	5	1.1.1.91.9	Winner

Protein Sequence Coverage - Protein-L-isoaspartate O-methyltransferase OS=Triticum aestivum GN=PCM PE=1 SV=1

MAQFWAEGSLEKNNALVEYLKQYGVVRTDKVAEVMETIDRALFVPEGFTPYTDSPMPIGYNATISAPHMHATCLELLKDYLQPGMHALDVGSGSGYLTACFAMMVGPEGRAVGIEHIPELVVASTENVERSAAAALMKDGSLSFHVSDGRLGWPDAAPYDAIHVGAAAPEIPRPLLEQLKPGGRMVIPVGTYSQDLQVIDKSADG
STSVRNDASVRYVPLTSRSAQLQDS

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General Appendices

Appendix 2: Reagents and protocols

Reagents

All reagents including the protein extraction, 2D cleaup, 2D sample rehydration buffer and 2D equilibration buffer were all kit based and purchased from Bio-rad.

Gel formulations

Pre-cast 12% criterion gels and Sypro-Ruby gel stain were purchased from Bio-rad

Destain/ fixing solution

After the second dimension, gels were removed and fixed with 40% MeOH, 10% glacial acetic acid for 30 minutes. Thereafter the gels were stained for 1hour on a rocker and destained with 40% MeOH, 10% glacial acetic acid overnight.

Appendix 4: Isoelectric focusing protocol for two-dimensional electrophoresis

Isoelectric focusing conditions for broad-range IPG strips rehydrated with total protein on a Bio-RadPowerPac^{TM} power supply

Step	Voltage	Ramp	Duration
1	250V	Rapid ramp	20min
2	8000V	Gradual ramp	1hr
3	8000V	Rapid ramp	29000VHS

Appendix 3: Reagents used for sample preparation and peptide identification

<u>i) ReadyPrep 2-DE Rehydration/sample buffer 1</u>

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

<u>ii) ReadyPrep TBN Reducing Agent</u>

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 -20oC to prevent evaporation of the TBP

- *1 ampoule contain 0.6mL of 200nmM tributylphosphine (TBP) in 1-methyl-2-pyrrolidinone sealed under nitrogen gas*

<u>iii) Prepare 3-5 dilutions of protein standard from 0.2mg/mL to 1.5mg/mL .</u>

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.

<u>iv) Preparation of Bovine serum albumin (BSA) stock (2mg/mL)</u>

0.002g of powder in 1000 μL of DH2O

= 0.01g of powder in 5000 μL of DH2O

Dilution of BSA for the standard curve

1mg/mL= 1mL of 2mg/mL + 1mL of DH2O

0.75mg/mL=0.75mL of the 1mg/mL dilution + 0.25mL of DH2O

0.5mg/mL = 0.5mL of the 0.75mg/mL dilution + 0.5mL of DH2O

0.25mg/mL= 0.5mL of the 0.5mg/mL dilution + 0.5mL of DH2O

0.125mg/mL= 0.5mL of the 0.25mg/mL dilution + 0.5mL of DH2O

0.0625mg/mL= 0.5mL of the 0.125mg/mL dilution+ 0.5mL of DH2O

0.03123mg/mL=0.5mL of the 0.0625mg/mL dilution + 0.5mL of DH2O

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 DH2O

1:100= 10 µL of the 1:10 dilution + 90µL of DH2O

1:1000=10 µL of the 1:100 dilution + 90 µL of DH2O

Preparartion of 1XSDS-PAGE gel running Buffer

-Prepare a 1XTris/glycine/SDS(TGS) running buffer by adding 100mL of the 10xTGS to 900mL of DH2O

OR

Prepare the 1X TGS from TGS powders as follows:

(25mM Tris, 192mM glycine, 0.1% SDA, PH 8.3

Preparation of Equilibration buffer I and II

NB) The equilibration buffers should be prepared about 15minutes before use. If the IPG strips were at -80oC 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 13.35mL of the supplied 30%glycerol solution. Each bottle contain 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 25-30oC as the solution stirs. Do not heat above 30oC.

Equilibration buffer II:

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

50mm NH4HCO3

-0.2g NH4HCO3 was dissolved in 50mL in H2O (NH4HCO3 Mw=79.06g/mol). The pH checked (it should between 7.5-8.5 and solution stored at 4Oc and used within 2 weeks.

5.5 mM CaCl2 in in 25mm NH4HCO3

-0.1g NH4HCO3 per50mL H2O + 0.03g CaCl2 (Mw=110.98g/mol) stored at 4°C

50mM NH4HCO3/50% MeOH (50mL) used within 2 weeks (stored at 4°C)

-0.2g NH4HCO3 to 25mL of MS grade H2O +25mL of 100% MeOH

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

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

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

-2.5mL MS-grade H_2O + 7.5mL ACN

Trypsin Stock

-200µl of the buffer supplied in the kit added to one vial containing 20µg trypsin and placed on ice

-the vial vortexed to dissolve the trypsin

-20µl aliquots in 0.5mL eppendorfs (work rapidly in an ice bath)

-The aliquots immediately stored at -20°C

Preparation of fresh 10ng/µl trypsin

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

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

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

Preparation of fresh 10mM DTT in 25mM NH_4HCO_3

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

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

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