EVALUATION OF VARIOUS PROTEOMIC TECHNIQUES TO IDENTIFY PROTEINS INVOLVED IN CEREAL STRESS RESPONSES TO APHID INFESTATION

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By

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DECLARATION

I NTOMBEKHAYA NQUMLA declare that this dissertation titled "Evaluation of Two Proteomic Techniques to Identify Proteins Involved in Cereal Stress Responses to Aphid Infestation" submitted for the award of the Master of Science 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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DEDICATION

This work is dedicated to my parents Kholisile and Nothobile Nqumla.

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ABSTRACT

All plants are exposed to abiotic and biotic stresses and have developed intricate signalling responses to survive. They respond to cell-structure disruption caused by herbivore probing and feeding by the formation of callose. Callose is a linear homopolymer made up of β-1,3-linked glucose residues with some β-1,6-branches. Plant responses to abiotic or biotic stress share events such as phosphorylation, membrane depolarization, calcium influx and the release of reactive oxygen species such as hydrogen peroxide. These events lead to the up-regulation of several pathways leading to biosynthesis of signalling molecules such as salicylic acid, jasmonate, abscisic acid and ethylene pathways.

The aim of this study was to determine the most suitable proteomic approach for identifying proteins and signalling pathways involved in cereal response to aphid infestation. An *in silico* approach was first evaluated in which the 5′ upstream regulatory region of proteins belonging to the family of callose synthases was scanned for *cis*-regulatory elements in order to identify which callose synthases are possibly expressed in plants during biotic or abiotic stresses. Bioinformatics tools were used in the identification of twelve *Arabidopsis* and ten rice callose synthase coding regions. Genome sequences for rice and *Arabidopsis* were scanned for the 2000 bp 5′ region upstream of the start codon of each callose synthase coding region. PlantCare, PLACE and Athena software were used to identify putative *cis*-regulatory elements present in the 2000 bp 5′ upstream sequences. The majority of *cis*-acting elements identified were involved in drought and high temperature responses and only one *cis*-acting element was involved in wound stress. These results therefore indicated a probable role for plant callose synthases in drought stress responses rather than in biotic stress responses. Genevestigator analysis of Arabidopsis results of micro-array experiments indicated that AtGSL10 is highly up-regulated, with AtGSL1, 3, 5, 6, 7, 8, 11 and 12 showing medium up-regulation and AtGSL2, 4 and 9 no up-regulation during aphid infestation of Arabidopsis plants, implicating a possible role for AtGSL10 in the plant response to aphid infestation.

An LC/MS/MS approach was used to identify specific signalling pathways involved in wheat resistance or stress response to aphid infestation. Eight proteins were identified as being up-regulated during aphid feeding 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 TugelaDN plants, were related to energy metabolism and located in the chloroplast.

Evaluation of two dimensional gel electrophoresis to identify phosphoproteins differentially regulated in wheat during aphid infestation, revealed the up-regulation of three proteins namely photosystem II oxygen-evolving complex protein 2, HVUNKNOWN from *Hordeum vulgare* subsp vulgare and HSKERAT9 NID from *Homo sapiens*. Additional 57 proteins were partially identified as involved in the stress response but due to low protein levels, the percentage of matching peptides to these proteins was below the required confidence level. Although these protein identifications were below the confidence level, it is interesting to note that several of the proteins are known stress response proteins, and therefore could serve as potential targets for future investigations.

In conclusion, the down and up-regulation of wheat proteins after aphid feeding reported in this study suggest that several signalling pathways are involved in the cereal stress response to aphid feeding. In *silico* approaches require knowledge or identification of potential proteins whereas 2D and LC/MS can identify numerous proteins still unknown to be involved in specific stress responses. The 2D approach is also limited in that the proteins of interest may be in low abundance and therefore not detected in the gels due to the presence of high abundant proteins. Therefore the best approach to identify proteins and signalling pathways involved in the stress response of wheat to aphid infestation, is the LC/MS/MS approach, as this proved to be the most sensitive and robust, identifying the most proteins with a high degree of confidence.

TABLE OF CONTENTS

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

LIST OF ABREVIATIONS

ABA- Abscisic acid

ACC-1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID

ADH- aldehyde dehydrogenase

AOC – allene oxide cyclase (AOC)

AOS - allene oxide synthase

APX- ascorbate peroxidase

cDNA - complementary DNA

CICR- Ca^{2+} induced $Ca2^{+}$ release

COR-cold regulated

CPR- constitutive repressor

CTR1- Constitutive Triple Response1

DAD1- defender against apoptotic cell death

EIN- ethylene-insensitive

ENO- enolase

ET- ethylene

ETR- ethyleneresistant

FNR- ferredoxin-NADP+-oxidoreductase

FQR- ferredoxin-plastoquinonereductase

FQR-dependent CET- ferredoxin-plastoquinonereductase-dependent cyclic electron transfer

GAPDH- Glyceraldehyde-3-phosphate dehydrogenase

HR - hypersensitive response

HSP- heat shock proteins

JA –jasmonic acid

LEA- Late embryogenesis abundant

LOX2 -lipoxygenase

$NADP^+$ - nicotinamide adenine dinucleotide phosphate

NDR- non-race specific disease susceptibility

NPR- non-expresser of PR genes

OEE1- oxygen-evolving enhancer protein

OPR3 – 12- oxo-phytodienoic acid reductase 3

PAD- phytoalexin-deficient

PGK3-phosphoglycerate kinase

PGM- phosphoglyceratemutase

PGR5- proton gradient regulation

PGRL1a- transmembrane protein present in thylakoids

PR- patgogen related

PSI - photosystem I

RNase – Ribonuclease

ROS- reactive oxygen species

RuBisCO- ribulose bisphosphate carboxylase/oxygenase

S - Sulfur

SA- salicylic acid

SAR-systemic resistance

SOD- superoxide dismutases

TFs – transcription factors

Trxh- Thioredoxin h

CHAPTER 1

LITERATURE REVIEW

1.1 INTRODUCTION

The major limiting factors in plant geographical distribution are environmental stresses, such as low or high temperatures, drought and salinity; which in turn affect plant growth and productivity. Plants are faced with a wide range of environmental stresses during their life cycle and therefore have developed mechanisms to increase their tolerance to various environmental conditions. They protect themselves through physical adaptations and interactive molecular and cellular changes that begin after stress initiation. The primary limitation to crop production is environmental stress, thus affecting crop production that leads to yield losses of more than 50% of major crops every year (Bushel *et al*., 2007). An extrinsic stress results from abiotic stress factors including temperature, drought, salinity, climate factors and chemical components; whether they occur naturally or are man-made; and is considered the most important stress (Qureshi *et al*., 2006).

1.1.1 ABIOTIC STRESS

Abiotic stress; in reference to plant science is defined as any environmental condition (non-living components) that can result in reduced plant growth, survival or productiveness. These include drought (water deficit), too high or too low temperatures, acidic or alkaline soil, high/low light levels and poor supply of nutrients (Boscaium *et al*., 2008). The above-mentioned factors occur in multiple stages and in most cases a single plant is exposed to more than one stress, resulting in decreased plant growth and development. These environmental stresses are major limiting factors in agriculture because all essential crops are sensitive to abiotic stress conditions, which lead to poor yield (Atienza *et al*., 2004). Abiotic stresses have gained much attention because of their potential impact on agriculture production and quality (Ligang *et al*., 2012).

1.1.1.1 DROUGHT

Drought stress is often associated with increased soil salinity; together these are major abiotic factors, affecting plant growth, development, survival and crop productivity. Therefore a good/detailed understanding of the complex mechanism of drought and salinity tolerance is essential for agricultural production (Ligang *et al*., 2012). Drought stress has been associated with leaf rolling in various plants including rice, maize, sorghum and wheat (Kadioglu and Terzi, 2007). Rice is more susceptible to water deficit than other plants, with its leaf rolling,

drying and yield reduction observed under drought conditions (Lian *et al*., 2006). Therefore, changes in cellular metabolism associated with osmotic adjustment were expected. Proteome changes during drought conditions have been intensively studied using the poplar as a model for plants (Lian et al., 2006).

(I) Drought responsive proteins

"Drought has been defined as one of the major limiting factors in crop production and it is becoming a severe problem in many regions of the world" (Passioura, 1996; 2007). Salekdeh *et al*., (2002) identified sixteen drought responsive proteins in rice, using a proteomic approach. Of the sixteen drought responsive proteins, only three were up-regulated by drought stress. These were S-like RNase homolog, actin depolymerisation factor and RuBisCO activase; while isoflavon reductase-like protein was down-regulated. In maize, nineteen drought responsive proteins were identified by Riccardi *et al*. (1998). Ke *et al*., (2009) reported the up-regulation of LEA-like proteins and chloroplast Cu-Zn SOD by drought stress, while Rieske Fe-S precursor protein was shown to be down-regulated. In rice and wheat, water deficit causes an increase in abundance of chloroplast Cu-Zn SOD (Salekdeh *et al*., 2002; Wu *et al*., 1999). In rice, when roots are subjected to stress, abs*cis*ic acid (ABA) is produced, which is then transported into aerial parts of the plant (Wu et al., 1999). It has been reported that down regulation of Rieske Fe-S precursor may lead to a decrease in photosynthesis. On the other hand ABA is known for causing significant changes in the photosynthetic proteins, for example: Rieske Fe-S precursor protein, and the accumulation of some defence/stress related proteins, which are responsible for the induction of stomatal closure (Rakwal *et al*., 2004).

In *Arabidopsis thaliana*, an up-regulation of genes encoding known components of ferredoxin-plastoquinonereductase-dependent cyclic electron transfer (FQR-dependent CET), PGR5 (proton gradient regulation) and PGRL1a (a transmembrane protein present in thylakoids) was reported upon drought stress (Lehtimäki *et al*. (2010). This suggests the induction of the FQR pathway in response to drought stress. Two genes that encode ferredoxin-$NADP^+$-oxidoreductase (FNR) isoforms were also reported to be up-regulated by drought stress at the transcriptional level. Lehtimäki *et al*., (2010) also reported the down regulation of two genes encoding two isoforms of PSAD, which is docking ferredoxin and PSI at both transcriptional and translational level.

ABA has been reported as the main chemical signal and the most important hormone, as far as drought is concerned, but other hormones such as cytokines and pH changes may also play an important role (Jia and Zhang, 2008). ABA is released in plants as in response to drought stress by inducing genes that encode enzymes and proteins involved in responses to cellular dehydration (Luan, 2002a; Zhu, 2002).

Bogeat-Triboulot *et al*., (2007) employed cDNA microarray and 2D-DIGE analysis in the investigation of parallel changes at transcript and protein levels in relatively drought-tolerant *Populuseuphratica*. Differences in gene expression upon drought stress at the transcription and protein levels were observed. Up-regulation of 1,4-α-glucan branching enzyme, Thioredoxin h (Trxh), alcohol dehydrogenase (ADH), cold regulated LTCOR12, asparagine synthetase, cysteine protease, trypsin inhibitors, xylose isomerise and sucrose synthase was observed at the transcription level in response to drought stress in *Populuseuphratica*. Up-regulation of several photosynthesis- and carbon metabolism-related proteins including ATP synthase β- subunit, ATPase α-subunit, RuBisCO activase, oxygen involving components, and proteins involved in glycolysis such as GAPDH and PGK were observed at a protein level in response to drought stress of *Populuseuphratica*. However there was down-regulation of several heat shock proteins (HSPs) and chaperons (Bogeat-Triboulot *et al*., 2007).

(II) Drought stress responsive transcription factors

Previous studies reported several transcription factors (TFs) that are up-regulated as a result of drought stress (Kizis *et al*., 2001; Xiang *et al*., 2008; Lu *et al*., 2007; Abe *et al*., 1997; Sakamoto *et al*., 2004; Wang *et al*., 2009). Most of the drought stress induced TFs belong to large plant gene families (Lorenz *et al*., 2011), such as AP2/ERF (Kizis *et al*., 2001), bZIP (Xiang *et al*., 2008), NAC (Lu *et al*., 2007), MYB (Abe *et al*., 1997), C2H2 zinc finger (Sakamoto *et al*., 2004) and WRKY (Wang *et al*., 2009). In *Arabidopsis thaliana*, NF-TB1 and NF-YA5 belonging to the CCAAT family were reported to be strongly associated to drought stress response (Nelson *et al*., 2007; Liu & Howell, 2010). The up-regulation of NF-Ya4 and NF-YB during drought stress response was reported in wheat (Stephenson *et al*., 2007), rice (Sarkar *et al*., 2009) and maize (Nelson *et al*., 2007). In *P. euphratica* an up-regulation of drought responsive transcriptional factors such as NF-YA4, NF-YA1, NF-YA3, and NF/YC11 was reported (Yan *et al*., 2012). These identified TFs were reported to have a regulatory role in ABA-dependent/ABA independent pathways during drought stress

(Shinozaki *et al*., 2003). In *P. euphratica* ABA-dependent transcriptional factors such as MYB, MADS, leucine zipper and C2H2 zinc finger were up-regulated at different drought stress intensity levels.

1.1.1.2 TEMPERATURE

In wheat; Rampino *et al*., (2012) reported the up-regulation of Td6TM1, which is a gene that codes for receptor protein homologous to putative receptor protein kinase, and which was found to be involved in plant defence and adaptation responses (Lehti-Shiu *et al*.,2009). Td4TF1, a homolog of putative t-complex protein 1 theta chain, was also up-regulated in a perennial grass during stress (Xu *et al*., 2010). A lipid modelling gene, a homolog of PER1-like protein gene, was involved in plant responses to variations in temperature but its involvement in abiotic stress is not fully understood (Chen *et al*., 1997; Lara-Avila *et al*., 2012). "Cold as a stress factor is associated with significant alterations in energy metabolism that causes a decrease in the rate of enzyme catalyzed 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 thalian*a (Bae *et al*., 2003; Amme *et al*., 2006), *Thellungiella halophila* (Gao *et al*., 2009), rice (Imin *et al*., 2004; Lee *et al*., 2009), chicory (Degand *et al*., 2009), meadow fescue (Cheng *et al*., 2010), pea (Taylor *et al*., 2005; Dumont *et al*., 2011) as well as poplar (Renaut *et al*., 2004) .

Cold is known to significantly affect photosynthesis. Korma *et al*., (2009) performed a study on proteomics response to cold (2ºC) using two genotypes of meadow fescue (*Festuca pratensis*) which differ in their frost tolerance. Significant differences were observed when protein abundance in these two genotypes exposed to cold was compared; the differences were between several components of a thylakoid-membrane-associated photosynthetic apparatus such as light-harvesting complexes, oxygen evolving complex, oxygen-evolving enhancer protein 1 (OEE1) and cytochrome b_6/f complex iron sulphur subunit or Rieske Fe-S protein. An up-regulation by cold has also been reported for stromal-located components of the Calvin cycle, especially for RuBisCO subunits and RuBisCO activase (Gao *et al*., 2009, Hashimoto & Komatsu, 2007). Studies have indicated changes in catabolic pathways and down-regulation of anabolic pathways. Up-regulation of enzymes catalyzing (terminal) steps of glycolysis such as triose phosphate isomerise glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 3-phosphoglycerate kinase (PGK), phosphoglyceratemutase (PGM) and

especially enolase (ENO) was observed. Cold-regulated/late embryogenesis-abundant (COR/LEA) proteins which are highly hydrophilic and can serve as high molecular osmoprotectants are also induced by cold stress.

Heat stress is associated with a greater risk of incorrect protein folding and denaturation of several intracellular proteins and membrane complexes. Several proteins are expressed in response to heat stress. Heat leads to enhanced accumulation several proteins with chaperone function, more especially members of the large the family of heat-shock proteins (HSPs) belonging to distinct sub-families according to their molecular weights (HSP100s, HSP90s, HSP70s, HSP60s, and sHSPS (HSPs <40kDa) (Trend, 1996). Up-regulation of HSPs plays a pivotal role in abiotic stress responses in plants (Sun *et al*., 2002; Sorensen *et al*., 2003; Wang *et al*., 2004). Transgenic plants overexpressing HSP genes exhibited improved tolerance to high temperature (Sanmiya *et al*., 2004). Up-regulation of several enzymes involved in redox homeostasis including dehydroascorbate reductase (DHAR), thioredoxin h-type (Trx h) and chloroplast precursors of SOD have been reported (Lee *et al*., 2007). An enhanced accumulation of enzymes involved in biosynthesis of UDP-glucose (UDP-glucose phosphorylase UGPase), biosynthesis of thiamine and dehydrogenation of pyruvate (pyruvate dehydrogenase) and transketolase was observed during heat stress, these enzymes are involved in energy metabolism (Lee *et al*., 2007).

The *Arabidopsis* SOS2 gene encodes a Ser/Thr protein kinase that is required in plant salt tolerance (Liu *et al*., 2000). Exposure to high salt stress may lead to SOS3–SOS2 protein kinase complex activation via calcium signals, which then stimulates the Na^+/H^+ exchange activity of SOS1 and regulates the expression of several genes at the transcription and post-transcription level (Zu *et al*., 2002).

1.1.1.3 Flooding stress

Flooding stress is considered the major condition amongst harsh environmental factors that have negative effects on plant growth, development and productivity. Flooding results in inadequate or blocked photosynthesis that leads to severe cellular reduction content (Bailey-Serres and Voesenek, 2008). The photosynthesis blockage requires ATP and NAD^+ generation which is produced through the anaerobic respiration. An insight into a low oxygen sensing mechanisms in metabolic alteration involved in controlled use of carbohydrate and ATP was provided by gene regulation evaluation and functions in model systems. Oxygen stress caused by flooding can be escaped by plants at the developmental stage through complex alterations in cellular and organ structure that promote access to and diffusion of oxygen (Fukao and Bailey-Serres, 2004). These processes are controlled by plant hormones such as abs*cis*ic acid, ethylene and gibberellin (Voesenek *et al*., 2003). However knowledge of adaptive mechanisms and response regulation at the transcriptional and post-transcriptional levels is not enough, the understanding of mechanisms underlying plant response to flooding is still limited (Kong *et al*., 2010).

Amongst proteins that were down-regulated during floods, two were related to the glycolytic pathway, one was fructose-1,6-bisphosphate aldolase and the other sucrose: fructan 6-fructosyltranferase (Kong *et al*, 2010). Triose phosphate has been described as the key carbon source involved in sucrose synthesis in plant tissues that participate in photosynthesis. This carbon source is derived from the Calvin cycle and moved from the chloroplast during the day. Fructose-1,6-bisphosphate aldolase catalyzes the conversion of two tyrose phosphates into fructose-1, 6-bisphosphate, a reversible reaction that occurs in the cytoplasm (Xue *et al*., 2008). Fructans, which are fructose polymers accumulate in cool-season grasses and cereals that are economically important are synthesized by sucrose:fructan 6-fructosyl transferase. It has been proposed that sucrose: fructan-6-fructosyltransferase might be critical for plant survival under stress conditions in those species in which fructans represent the major form of server carbohydrate (Lasseur *et al*., 2009). Fructose-1,6-bisphosphate aldolase and sucrose:fructan 6-fructosyl transferase are down-regulated by flooding stress(Lasseur et al., 2009). This supports the idea that photosynthesis inhibition by flooding stress results in the reduction of carbohydrate metabolism and energy consumption in wheat seedlings. Thus wheat seedlings exposed to long-term flooding can adjust their metabolic status and reserve energy to cope with the flooding stress. Proteins that are related to cell wall structure and modification account for a major proportion of the proteins that are down-regulated by

flooding. These include methionine synthase, β-1,3-glucanases, β-glucosidase, β-galactosidase and glucanase precursor (Kong *et al*., 2010).

1.1.1.4 CLIMATE CHANGE

Whether naturally occurring or man-made, climate change is the greatest challenge worldwide nowadays. Increases in carbon dioxide, methane and nitrous oxides are a consequence of fossil fuel combustion and are responsible for most of the increase in global temperature levels that were observed since the middle of 20^{th} century, as reported by International Panel on Climate Change (IPPC) (2007). These factors affect plant growth, development and function, starting with photosynthesis, the most important process in plant (Drake *et al*., 1997). CO_2 is a substrate and a fundamental C-source for photosynthesis in plants, where other biomolecules originate (Drake et al., 1997). Fixation is mediated by RuBisCO, which is the most abundant enzyme in nature and constitutes more than 50% of the total soluble proteins in leaves. An increase in CO_2 results in an instant increase in the rate of photosynthesis in C3 plants, including rice, which gradually lowers as the exposure to high CO_2 is prolonged because of acclimation and for the plants to achieve homeostasis (Kim, 2009). Huge amounts of CO_2 from the atmosphere are sequestered by photosynthesis. "The sink effect is of extraordinary importance because it can counteract the present trend towards a rise atmospheric CO_2" (Lenton and Huntingford, 2003). Extreme temperature and increased incidence of environmental stress such as drought and salinity, together with increased CO_2 levels will probably establish the greatest risk caused by global climate change to agricultural ecosystems in arid or semi-arid areas of the world (Luo and Mooney, 1999; Centritto *et al*., 2002).

The effect of increased CO_2 levels on plant growth and crop yield has been well studied using different methods such as greenhouses, inter alia, indoor growth chambers, field tunnels, and closed or open-top field chambers (Ainsworth and McGrath, 2010). Free air CO_2 enrichment (FACE) techniques have been developed and applied (McLeod and Long, 1999; Hendry and Miglietta, 2006). The FACE technology leaves the natural environment of a crop plot nearly fully unperturbed and is currently regarded as the most realistic CO_2 exposure system.

Studies by Ainsworth and McGrath (2010) reported the calculated yield enhancements of globally important crops wheat, soybean and rice to be up to ca. 30% at a CO_2 concentration of 550 ppm. These results were based on the compilation of published crop responses

because of elevated CO_2. However some reported estimated effects of increased CO_2 levels on crop yields that were obtained using enrichment techniques other than FACE as ranging between 28% and 35% (Amthor, 2001; Jablonski *et al*., 2002). Arguments that growth and yield responses of crops to elevated CO_2 may be lower under real field than yields obtained under idealized growth conditions were raised (Long *et al*., 2005, 2006).

FACE studies conducted in the past 15 years at 550 ppm CO_2 levels showed the crops yield (wheat, rice, soybean, potato) only increased by 13% to 17% compared to crop yields that are obtained under current levels of CO_2 (Kimball *et al*., 2002; Long *et al*., 2006). Crop modeling results and projections of future global food production under climate change show that crop yield depend on the integration of the CO_2 effect and the fertilization effect (Parry *et al*., 2005; IPCC, 2007; Lobell *et al*., 2008; Lobell and Burke, 2010).

Global warming and climate change are a result of increased greenhouse gases such as CO_2, methane and Ozone (O_3). IPCC (2002) has reported that the CO_2 concentration is to double compared to the current level by the end of this century. Increased levels of CO_2 may indirectly improve photosynthetic response of wheat by elevating the atmospheric temperature as showed by up-regulation of RubBisCo-limited photosynthetic rate at higher temperatures (Alonso *et al*., 2009). Plant response mechanisms to increased levels of CO_2, have not been fully understood, however recent proteomic study has revealed that some enzymes are down-regulated in the regeneration phase of the Calvin cycle in rice plants grown under high CO_2 conditions (Bokhari *et al*., 2007).

C3 plants such as soybean, rice and wheat have shown greater sensitivity to atmospheric CO_2 than C_4 crops such as sugar cane, maize and sorghum. C4 crops have a 10-fold higher CO_2 assimilation rate, have a low water demand and have a more effective CO_2 capture system under extreme temperature conditions as compared to plants that belong to the C3 family. Attemps towards introduction of C4 photosynthesis pathways into C3 plants are still in progress to cope with predicted higher average temperature and more frequent extreme weather events and drought (Sheehy *et al*., 2000; Taniguchi *et al*., 2008).

O_3 is the most toxic photochemical component of all air pollutants and has been reported as being the cause of significant damages in crop plant; (Kley *et al*., 1999). Maize and bean proteomic responses to acute O_3 (0.2 ppm) showed that the up-regulation of antioxidants defence proteins SOD, APX and GSTs was a common feature for both plants (Akiko *et al*., 2010).

1.1.2 Biotic stress

Biotic stress is the stress that is caused by insects or herbivores, pathogens including bacteria and fungi (Haung *et al*., 2011). Plants have developed different defence mechanism to protect themselves from biotic stress. These lines of different defence mechanisms often overlap with each other (Valcu *et al*., 2009).

1.1.2.1 INSECTS

Plants have evolved several defence mechanisms to protect themselves against insect herbivores (Buell, 1998). Defences such as physical barriers and antibiotic compounds are synthesized as a result of attack by insects. Induction mechanisms are switched on in plants in response to wounding by herbivores or pathogens (Yuan *et al*., 2004). Induced defence mechanisms have a major role in conferring resistance or tolerance against various biotic stresses and may also have benefits for plants that lack constitutive defences (Maleck & Dietrich, 1999). Up-regulation of plant defences to insect feeding is highly dependent on the wound-response signalling (Thaler, 1999; Reymond *et al*., 2000).

Resistance in tomato that is induced in response to herbivores is very much dependent upon the octadecanoidor-jasmonic acid (JA) pathway (Howe *et al*., 1996; Li *et al*., 2004; Thaler *et al*., 2002). Rawat *et al*., (2013) reported the up-regulation of NBS-LRR, Cytochrome P450, heat shock proteins, phenylalanine ammonia lyase and OsPR10a genes from the Suraksha library, obtained from real-time PCR, and that R gene mediated salicylic acid related defence pathways may also be involved in gall midge resistance. Resistances in Suraksha against gall midge had a similar nature to the resistance observed in plants against pathogens. However, an up-regulation of TN1 genes related to primary metabolism and redox homeostasis was observed, suggesting that genes encoding translationally controlled tumour protein and NAC domain proteins are likely to be involved in the gall midge susceptibility. Studies have reported the up-regulation of trichome development in new leaves resulting from chewing organisms (Dalin *et al*., 2008). Ethylene (figure 1.2) and jasmonic acid (JA) (figure 1.1) are the two most important wound-response signalling molecules in plants (*Penninckx et al.*, 1998). Secondary metabolites and other proteins that are up-regulated after insect wounding and JA treatment often affect insect feeding, growth and development and attract plant predators (Pare & Tumlison, 1999; Walling, 2000). Yuan *et al*., (2004) has reported that Brown plant hopper (BHP) infestation on rice causes the up-regulation of the rice gene (BpHi008a), a gene which is downstream of the ethylene pathway.

1.1.2.2 HERBIVORES

The damage that is caused by herbivores modifies the physiology of the plants thereby resulting in the induction of signal responses (Agrawal, 1998). Studies have reported the induction of signalling pathways by herbivore wounding and saliva of the herbivores (Ryan and Moura, 2002; Stratmann 2003; Zavala *et al*., 2004). Herbivores induce the up-regulation of phytoalexins (Urani *et al*., 1975; Loper, 1968; Akanzwa *et al*., 1960). These antimicrobial compounds (Kuc & Rush, 1985) are mostly present in extremely low concentrations before infestation. The damage that is caused by herbivores in plants has an effect in plants nitrogen concentration and other nutrients that are vital in foliage (Benz, 1974). Studies have shown that phytoalexins are also active against insects and pathogens feeding on plants. Herbivore probing on plants results in local and systemic induction of volatile emissions (Turlings *et al*., 1995; Paré and Tumlinson, 1997; Dicke *et al*., 1999; Thaler *et al*., 2002). Volatiles are synthesized by many plants, as a form of direct or indirect defence against herbivores (Langenheim, 1994; Karban and Baldwin, 1997; Tholl, 2006).

Studies that have been done on soybean show that vegetative storage proteins (VSPs) VSPβ and VSPα are up-regulated in the stems and other vegetative tissues in response to attacks by herbivores (Stastwick *et al*., 1994). Two genes (*AtVSP1* and *AtVSP2*) that were highly similar to soybeans VSP and phosphatases were up-regulated in *Arabidopsis* after herbivore wounding (Berger *et al*., 1995; 2002). In *Arabidopsis* these genes are regulated by the jasmonate pathway (Berger *et al*., 1996). The interaction between JA and ethylene results in *Arabidopsis* resistance against wounding by herbivores (Rojo *et al*., 2003). Permine, which activates WIPK and SIPK, was also up-regulated in tobacco leaves (Takahashi *et al*., 2003) and a S-adenosylmethionine (SAM) decarboxylase gene, which is involved in polyamine synthesis, was up-regulated as a result of spider mite infestation of Lima bean leaves (Arimura *et al*., 2002). In maize, damage by herbivores, results in the induction of terpenes and volatile indole (Albon *et al*., 1997), which is synthesized by *ZmIgl*, a gene which encodes indole-3-glycerol phosphate lyase (Frey *et al*., 2000). *ZmIgl* in turn is up-regulated upon wounding by herbivores (Frey *et al*. 2000). Up-regulation of indole upon herbivore wounding was also confirmed in rice (Zhuang *et al*., 2012). Wounding by herbivores results in the up-regulation of defence proteins such as protease inhibitors (PI), polyphenoloxidase (PPO), lipoxygenase (LOX), α-amylase inhibitors, chitanase, β-1,3-glucanases and other proteins that are pathogen related, for their protection (Heil *et al*., 2006).

1.1.2.3 PATHOGENS

Plants have developed a defence mechanism that protects them against various pathogen infections (Kano *et al*., 2011). A plant′s successful defence against pathogenic infections depends on the rapid recognition of the pathogen′s attack and up-regulation of appropriate defence mechanisms (Ebel and Cosio, 1994; Jones and Takemoto, 2004) such as phytoalexins, reinforcement of plant cell walls, reactive oxygen species (ROS) production as part of the hypersensitive esponse, 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).

Up-regulation of two pectin related genes *PMR5* (whose function has not yet been identified) and *PMR6* (which codes for a putative glycosylphosphatidyl inositol –enchored pectate lyase in *Arabidopsis thaliana*) were described in a study screening for resistant mutants to pathogens (Vogel *et al*., 2002, 2004). Both *PMR5* and *PMR6* had an effect on pectin composition and were needed for *Arabidopsis* susceptibility to biotrophic powdery mildew pathogens such as *Erysiphe cichoracearum* and *Erysiphe orontii*. Inoculation of plants with a virulent/avirulent strain of pathogen leads to the plant′s resistance or susceptibility responses (Katagiri *et al*., 2002). In either of these case, the initial response is the activation of microbe associated molecular patterns (MAMPS) which induce signalling events such as calcium ion fluxes, ROS or nitric oxide (NO) synthesis, activation of mitogen-activated protein kinases (MAPKs), Ca^{2+}-dependent protein kinases (CDPKs or CPKs) as well as transcription factors such as WRKY22 and WRKY 29 (Angle *et al*., 2006; Yoshioka *et al*., 2011).

Biotic elicitors such as oligosaccharides that are derived from the cell walls of various fungi, bacteria, yeast and even marine algae, have been reported to induce systemic resistance (Aziz *et al*., 2004; Boller, 1995; Côté and Hahn, 1994; Darvill and Albersheim, 1984; Ebel and Cosio, 1994; Inui *et al*., 1997; Nakamura *et al*., 1999; Pauw *et al*., 2004; Agrawal *et al*., 2002).

Viruses, which are phloem restricted, are transmitted by homopteran insect taxa, including aphids (Aphididae) and whiteflies (Aleyrodidae). Plant viruses belonging to the families of *Luteoviridae* and *Geminiviridae* cause serious economic loss in wheat, potato, maize, sweet potato and cassava. Viruses are normally transmitted by one vector taxa (Nault and Ammar 1989; Legg *et al.*2002; Gray and Gildow 2003; Li *et al*., 2010). *Barley yellow dwarf virus (BYDV), Cereal yellow Dwarf virus (CYDV), Potato leafroll virus* and *Beet western yellows*

virus all belong to the *Luteoviridae* family of viruses (collectively referred to AS luteovirids) (Yang *et al*., 2008). They are transmitted only by aphids and involve a number of different proteins (Mayo & D′Arcy, 1999).

Aphids obtain plant viruses from infected phloem cells while feeding with their stylets. These viruses are sucked out of the plant into the aphid′s gut lumen and traverse the aphid′s midgut lining or hindgut to gain excess into the body cavity, and then circulate into the hemolymph (Garret *et al*., 1993, Gildow, 1993, Reinbold *et al*., 2003). Virons are then transmitted into the accessory salivary gland cells by endocytosis where they are transported into the salivary duct to be transmitted into a new host plant. *Luterovid* species are transmitted by a specific set of aphids, thus showing high vector specificity (Yang *et al*, 2008). The specificity of the vectors is regulated by the interaction between two aphid virus structural proteins and unknown proteins (Gray and Gildow, 2003). Studies have reported rapid accumulation of colnelenic acids (divinyl ethers originating from C18:2- and C18:3-derived hydroperoxides, respectively, via CYP74D catalysis) when the potato leaves are inoculated with either virus or fungi.

Fungi are known to be largest group of pathogens that belong to the *Ascomycete* and *Basidiomycete* classes (Lus *et al*., 2003). These pathogens have been reported to have developed sophisticated ways to penetrate the surface of the plant, including specialized infection structures and a repertoire of enzymes capable of digesting the external layers of host (Park *et al*., 2008). Parasitic fungi of aquatic plants, including *Endomycetes*, particularly species of the genus *Doassansia* and *Tracya* are well known (Batko, 1975). The knowledge of saprophytic mycoflora of this group of plants is only fragmentory (Voronin, 1992). Signalling intermediates such as jasmonic acid (JA), salicylic acid (SA) and ethylene increase their concentrations after pathogen infection, thereby playing key roles in signalling for defence (Glazebrook, 2005). Exogenous application of these compounds induces defence-related gene expression (Schenk *et al*., 2000).

Systemic acquired resistance up-regulation is triggered by pathogen infection, salicylic acid, probenazole or benzothiadiazole-S-methyl ester (BTH) (Gaffney *et al*., 1993; Gorlach *et al*., 1996; Nakashita *et al*., 2002). Studies have reported that infestation of rice plants by D-psicode results in the up-regulation of multiple defence related PR-protein genes (Kano *et al*., 2011). The hypersensitive response (HR) has been reported as the most efficient defence

mechanism, which induces quick physical changes, stopping pathogen multiplication inside the host cell (Greenberg & Yao 2004).

Studies have also revealed the important role played by ROS in the up-regulation of viral-stimulated signalling pathways (Gonzalez-Dosal *et al*., 2011). ROS can be produced in different cell compartments, thereby creating a local gradient for oxidising the proteins that are close by (Woo *et al*., 2010). The protein function is therefore modified by oxidation which disrupts the protein complexes thus inducing protein post-translational changes, which lead to the up-regulation of signalling pathways (Gonzalez-Dosal *et al*., 2011, Liu *et al*., 2000, Mieyal *et al*., 2008).

1.1.2.4 APHIDS

Aphids (*Sternorrhyncha aphididae*) are known as phloem feeders and are known for causing severe loss in cultivated plants worldwide (Powel *et al*., 2006). This is due to their highly efficient colonization and settlement, which is the result of several biological characteristics. Secondly, winged adults colonize new host plants whereas wingless adults invest more resources in reproduction (Powell *et al*., 2006). Thirdly, high population densities of aphids lead to significant nutrient removal from sieve tubes and lastly, they serve as vectors for several phytoviruses.

Plants have developed molecular and physical defences against herbivore attacks, either constitutive or inducible. As a result most herbivores leave damaged plants to exploit naive or healthy ones. Aphids, however, remain on a plant for longer periods and even induce leaf rolling for protection. Aphid survival is dependent on their ability to access phloem bundles while avoiding or sabotaging plant defence responses, thereby allowing them to remove their liquid diet while keeping the phloem cells alive (Tjallingii, 2006). "In the initial insertion of aphid stylets in epidermal tissues up to the prolonged feeding on sieve tubes, aphids continuously inject salivary secretions within plant tissues" (Goggin, 2007). These saliva injections are thought to contain substances required to counteract plant defence (Goggin, 2007). Regardless of their furtive strategy, phloem feeders are capable of causing alteration in their host plant, including morphological changes, modified resource allocations and various local as well as systemic symptoms (Goggin, 2007).

Plant responses to phytopathogenous insects or pathogen attack share common events, for example protein phosphorylation, membrane depolarization, calcium influx and release of reactive oxygen species such as hydrogen peroxide (Garcia-Brugger, 2006). These events

lead to phytohormone-dependent pathway activation as well as ethylene (ET) and jasmonate (JA) dependent responses; these being activated normally by necrotropic pathogens (Thomma *et al*, 2001) and grazing insects (Maffei *et al*, 2007). Salicylic acid (SA) dependent responses are triggered by biotropic pathogens (Thomma *et al*, 2001). On the other hand plants are able to fine tune differential production of SA, JA and ET signalling molecules to adapt their response to the type of bioagressor (deVos *et al*, 2005; Pieterse & Dicke, 2007). Plant defences against sucking insects such as aphids are 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 defence 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).

1.1.3 SIGNALLING PATHWAYS INVOLVED IN ABIOTIC AND BIOTIC STRESS

1.1.3.1 JASMONIC ACID

JA comes from a group of compounds known as oxylipins that are produced via oxygenation of fatty acids. The synthesis of JA commences in the chloroplasts where lipoxygenases convert linolenic acid into hydroperoxylinolenic acid. JA, together with related compounds, are involved in the regulation of plant responses to wounding and necrotrophic pathogens (Devoto and Turner, 2005). Jasmonate synthases also regulate pollen maturation and wound responses in *Arabidopsis*. The defence of function JA was first reported by Farmer and Ryan, (1992), who provided evidence that there is a link between wounding caused by insects, JA formation and protease inhibitor genes that prevent insect feeding. They also proposed that wounding causes the formation of linoleic acid, which is the alleged precursor of JA.

JA-mediated reprogramming of gene expression is best characterized in relation to plant-pathogen interactions (Overmeyer *et al*., 2003; Pauwels *et al*., 2009). JA and ET can act synergistically in the regulation of defences against necrotrophic pathogens and herbivorous insects. JA and ET-dependent defence pathways are activated by c insects and necrotrophic pathogens (Kerchev *et al*., 2012). JA signalling can be induced by a wide range of biotic stresses, including osmotic stress (Kramell *et al*., 1995), exposure to elicitors (oligosaccharides and oligogalacturonides), drought (Doares *et al*., 1995) and yeast extracts

(Parchmann *et al*., 1997; Leon *et al*., 2001). Parthier *et al*., (1992) provided evidence that jasmonates are involved in the regulation of gene expression, by observing the accumulation of jasmonate inducible proteins (JIPs) in senecing barley leaves (Weidhase *et al*., 1987; Mueller-Uri *et al*., 1988). Farmer and Ryan, on the other hand demonstrated the induction of the accumulation of protease inhibitors by MeJA and jasmonic acid as a direct defence against insect herbivores (Farmer and Ryan 1990, Farmer *et al*., 1991).

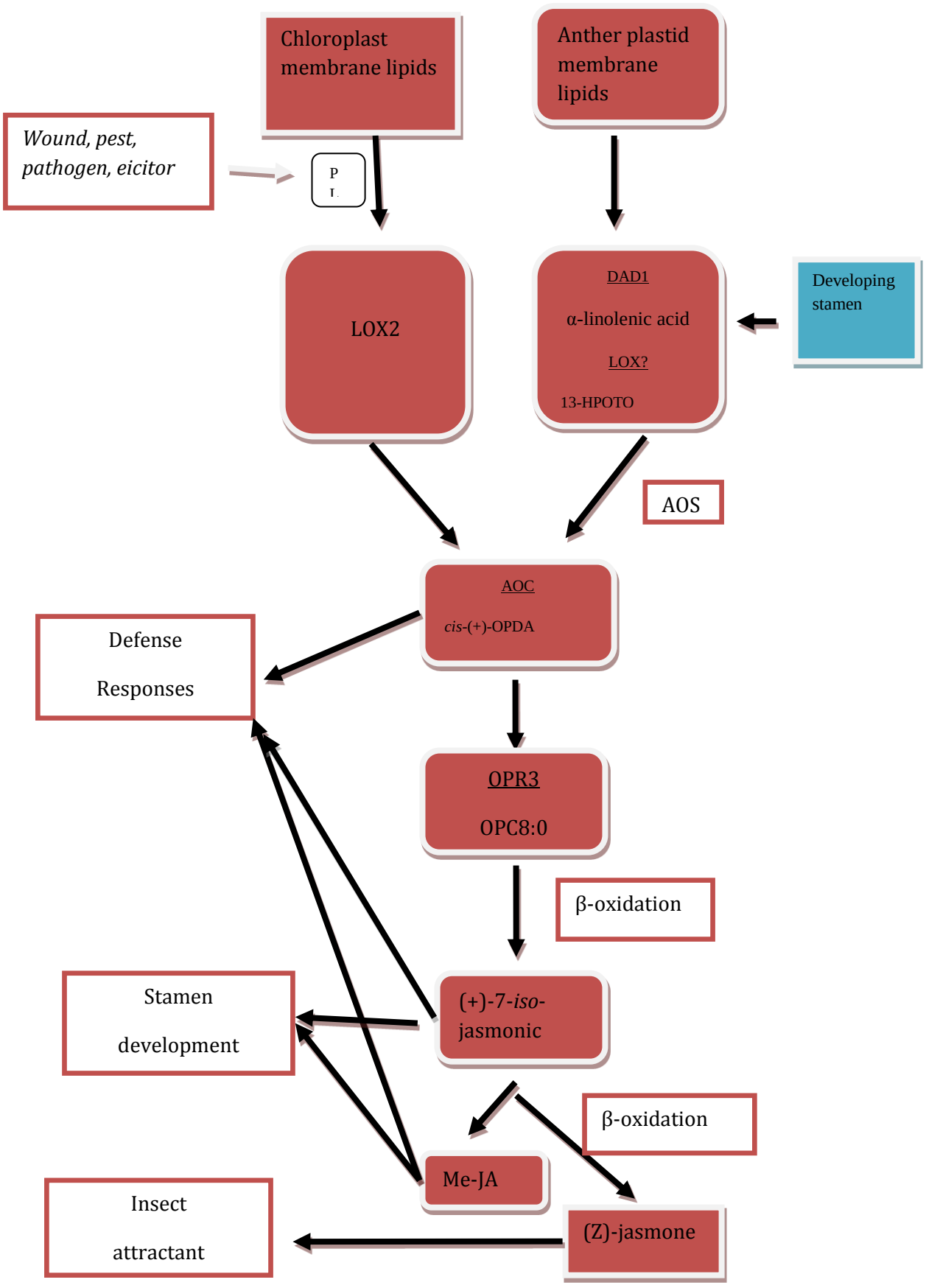

Figure 1.1: The model biosynthesis of jasmonic acid adapted from Turner *et al*., (2002).

1.1.3.2 ETHYLENE

Ethylene is known as a gaseous plant hormone that is synthesized in response to stress or pathogen attack, but also affects a myriad of developmental processes, plant germination, leaf abs*cis*sion, root nodulation and programmed cell death (Johnson & Ecker, 1998; Bleecker and Kede, 2000). In *Arabidopsis*, signalling ethylene is made of a group of membrane-associated receptors such as ETR1/ERS2, ethylene response sensor Ethylene receptor1 (ERS1)/ERS2 and the ethylene insensitive 4 (EIN4) component (Chang *et al*., 1993; Sakai *et al*., 1998). These receptors can only be active in the absence of ethylene, resulting in the activation of Raf-like serine/threonine (Ser/Thr) kinase and Constitutive Triple Response1 (CTR1) which are negative regulators of the ethylene pathway (Hua & Meyerowitz, 1998; Kieber *et al*., 1993). 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). Ouaked *et al*. (2003) identified several Medicago MAPK kinases that are up-regulated during abiotic and biotic stress; these were particularly SALT-STRESS-INDUCIBLE MAPK (SIMK) and Medicago MAPK3 (MMK3) which were activated by MAPKK (SIMK KINASE) during salinity treatment and pathogen elicitor treatment (Cardinale *et al*., 2000).

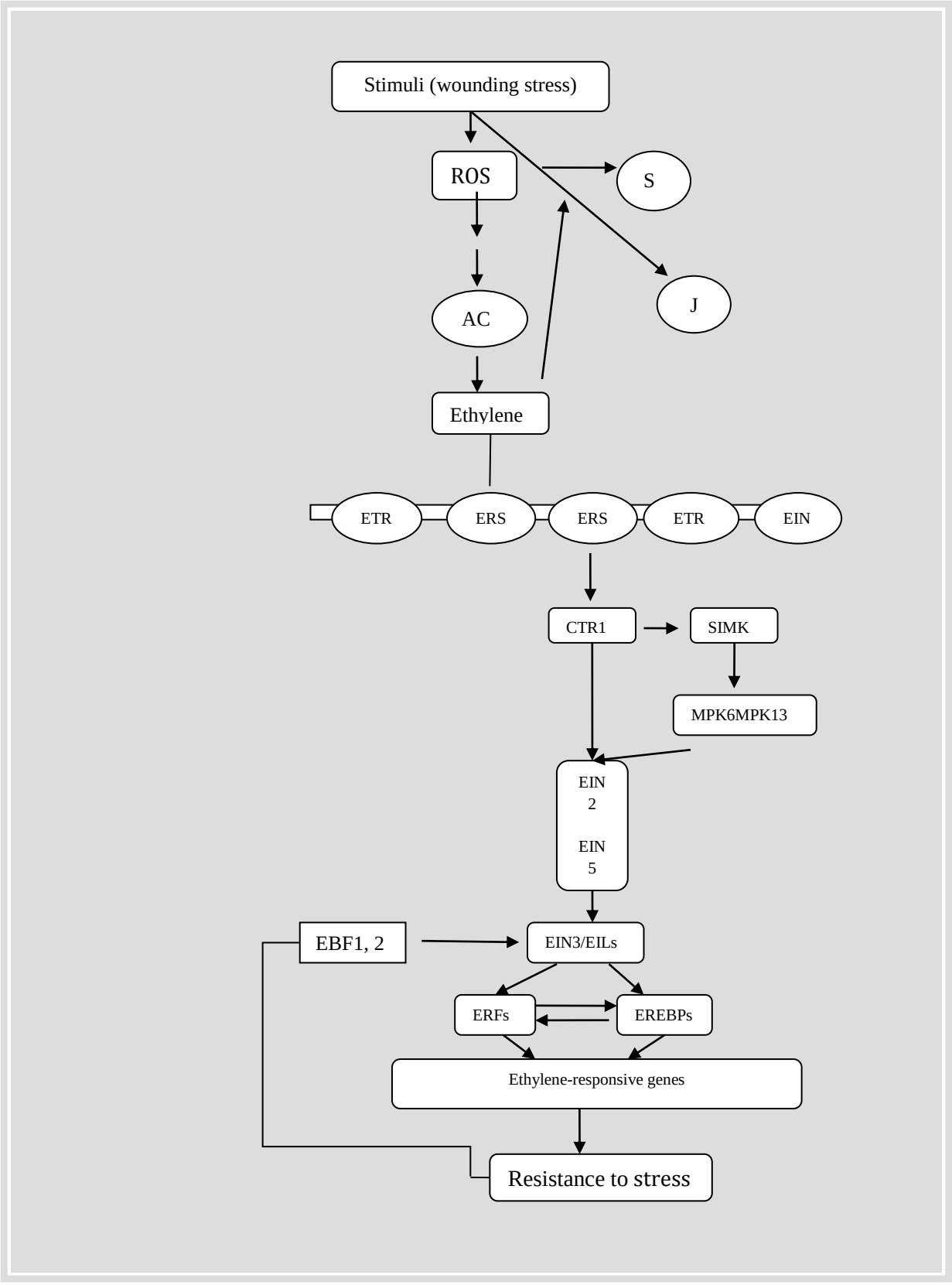

Figure 1.2: Signal transduction mediated by ethylene adapted from Tian and Yang (2006).

1.1.3.3 ABSCISIC ACID (ABA)

ABA is a positive regulator of leaf senescence that accumulates in response to stresses that involve water deficits, such as drought, salt or extreme temperatures, and lead to a reprogramming of gene expression and adaptive responses such as stomatal closure and the buildup of osmo-compatible solutes (Chandrasekhar *et al*., 2000). Overexpression of rice DSP, OsPFA-DSP1 in tobacco increased sensitivity to ABA-induced stomatal closure and inhibition of the opening of the stomata (Liu *et al*., 2012). It indicated that reversible protein phosphorylation plays an important role in regulating plant responses to abiotic stress (Li *et al*., 2012). Studies indicated that increased ABA synthesis and MAPKs signalling cascades play important roles in stomatal movement under drought stress (Jammes *et al*., 2009; Xiong and Yang, 2003). It has been reported ABA-induced stomatal closure was impaired in RNA lines of both AtMPK3 and ArMPK9 in *Arabidopsis thaliana* (Jammes *et al*., 2009).

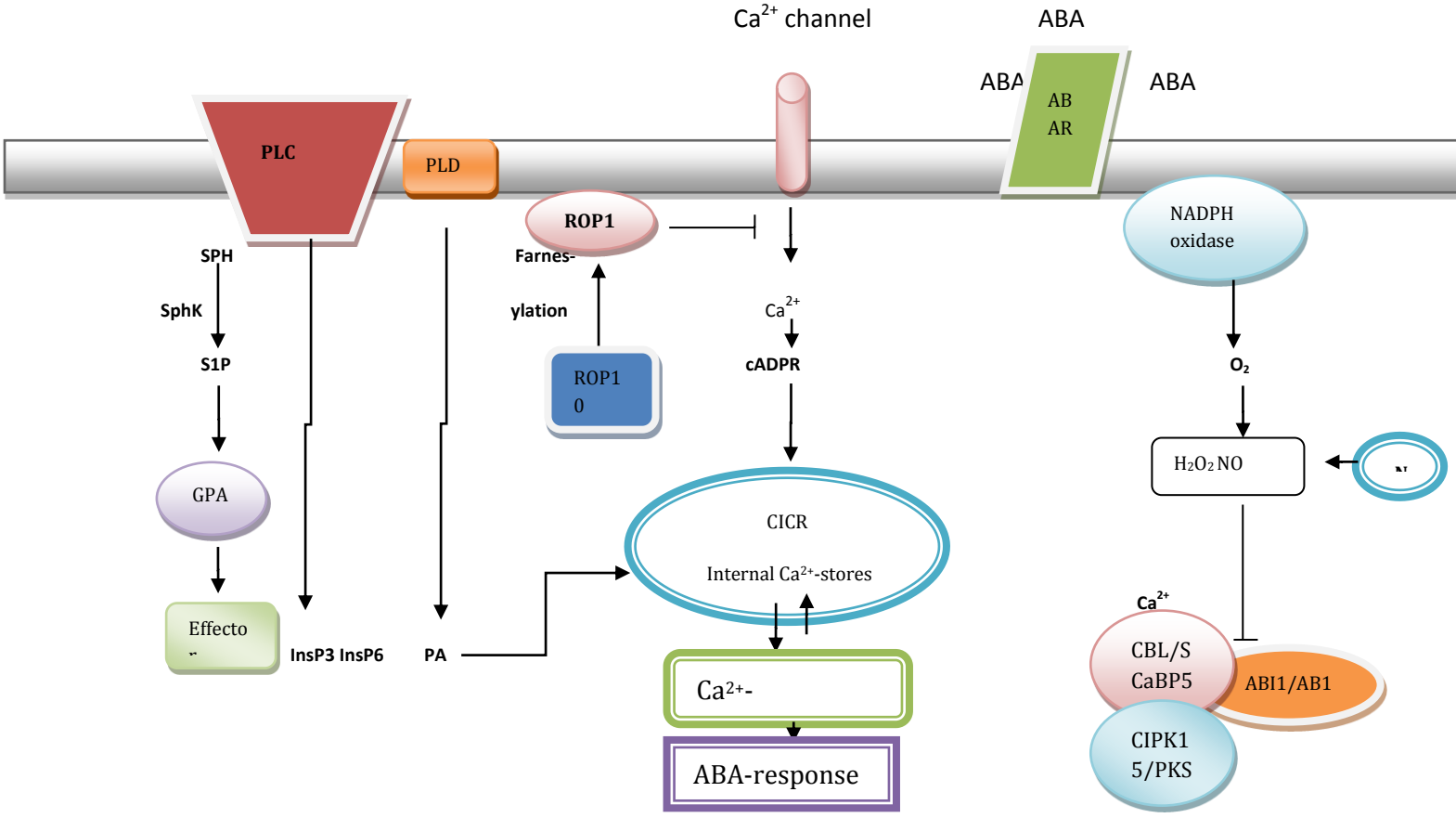

Figure 1.3: A schematic overview of signals controlling ABA responses in the guard cells. Adapted from Himmelbach *et al*. (2004)

1.1.3.4 Salicylic acid

Salicylic acid (SA) is a phenolic phytohormone that is needed in the signal transduction cascades that regulate plant defence mechanisms against abiotic and biotic stresses. It is essential in particular in systemic acquired resistance (SAR), which is known as a broad-spectrum plant immune response involving profound transcriptional reprogramming (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 reprogramming of 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 and accompanying cytosolic $Ca2^{+}$ release that takes place in the apoplast in response to the perception of abiotic and biotic stress (Kawano & Muto, 2000). The apoplastic oxidative burst and accumulation of resultant ROS in the extracellular space is characteristic of plant cells that are exposed to physical and chemical shock, insects and herbivores, symbiotic microorganisms and pathogens (Carlos *et al*, 2012). NADPH oxidase activation serves to restrain the spread of pathogen- and SA-induced cell death (Pogány *et al*., 2009).

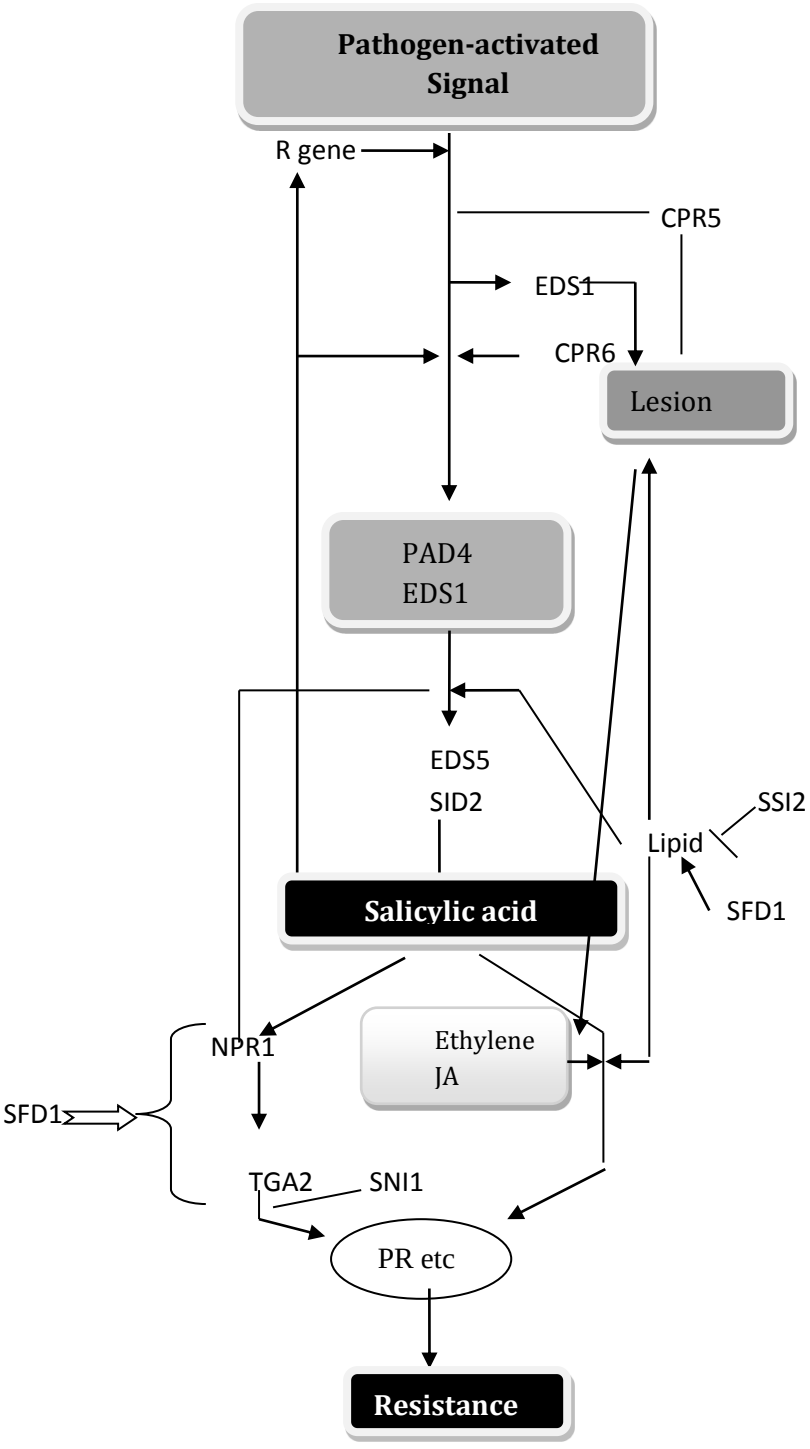

Figure 1.4: A schematic diagram of the salicylic signalling network. Adapted from Farmer *et al*., 2003.

1.1.4 Cross tolerance

Cross-tolerance to environmental stresses is an ordinary phenomenon in plants, whereby exposure to one type of stress confers a general increase in resistance to a range of different stresses (Pastori & Foyer, 2002; Suzuki *et al*., 2012). Cross-tolerance takes place because of synergistic co-activation of non-specific stress responsive pathways that cross biotic stress boundaries (Bostock, 2005). Cross-tolerance phenomena are always linked to the enhanced production of reactive oxygen species (ROS) such as H_2O_2, oxidative signalling and the associated regulation of gene expression through the redox signalling hub (Bartoli *et al*., 2012). H_2O_2 and other ROS are important signalling molecules in abiotic and biotic stress responses, due to the fact that they serve as messenger for activation of defence genes (Foyer and Noctor, 2009, 2012). Firm spatio-temporal control of redox signalling molecules tolerate different and sometimes diametrically opposed physiological events, thus generating signal specificity that is integrated with the action of plant hormones such as ethylene (ET), salicylic acid (SA), abs*cis*ic acid (ABA) and jasmonate (JA) (Xiong *et al*., 2002; Glazebrook *et al*., 2003; Fujita *et al*., 2006). The exposure to the atmospheric pollutant ozone generates ROS in the apoplast of the plant cell, thus initiating an oxidative signalling cascade that shares many signalling and regulatory response components with ROS mediated responses to abiotic and biotic stresses (Baier *et al*., 2005).

There has been evidence of cross talk between the hormone-induced defence-signalling pathways and redox signalling pathways that might be of vital importance in defining the plant defence strategy, depending on the type of the attacker (Kerchev *et al*., 2012). The increased ozone sensitivity of JA mutants supports the opinion that JA contributes to the containment of the ROS-dependent lesion propagation. In addition, ET-dependent lesion propagation may be influenced by JA by reducing the ET-dependent ROS generation (Overmeyer *et al*., 2003). SA is the key compound for the regulation of fungal, bacterial and viral pathogen resistance. It provides a signal for expression of pathogenesis-related proteins and other potentially defence factors induced after pathogen attack (Beckers and Spoel, 2006). On the other hand jasmonic acid produced in the octadecanoid pathway via lipoxygenation of linolenic acid, serves as a signal for expressing of a number of proteins including polyphenol oxidase and proteinase inhibitor, involved in plant defence against many attacks by insects (Bostock, 2005). There have been suggestions that SA and JA pathways are antagonistic to each other (Beckers and Spoel, 2006; Bostock, 2005; Pena-Cortés, 1993) and evidence has been reported that SA signal can strongly inhibit JA-

dependent defence signalling (Felton *et al*., 1999). However JA can be a potent inhibitor of SA-dependent signalling (Cui *et al*., 2005). It has been suggested that the SA pathway may be exploited by herbivores to reduce jasmonate-dependent response expression (Bostock *et al*., 2001).

1.2 PROBLEM STATEMENT

South African wheat farmers spend millions of Rands on pesticides and herbicides to produce two million tons of cereals/year. Aphid feeding on cereal plants can lead to extensive crop damage and serious loss of yield. Climate changes have been shown to increase aphid population. The development of new aphid resistant cereal cultivors, therefore, is crucial to the national and international cereal industry.

1.3 HYPOTHESIS

A proteomic approach will identify differentially regulated proteins involved in the cereal stress responses to aphid infestation.

1.4 AIM OF THE STUDY

To identify which proteomics approach is most suitable for identifying proteins and signalling pathways involved in cereal response to aphid infestation.

1.5 OBJECTIVES

- Evaluation of an *in silico* approach using callose synthases as a model protein family
- Evaluation of a LC-MS/MS approach to identify differentially regulated proteins or phosphorylated proteins in plant response in wheat to aphid feeding
- Evaluation of a two-dimensional gel electrophoresis approach to identify differentially regulated or phosphorylated proteins in plant response in wheat to aphid feeding

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CHAPTER 2

A TARGETED APPROACH TO IDENTIFY POSSIBLE INVOLVEMENT OF APHID FEEDING SPECIFIC PROTEINS IN PLANTS USING AVAILABLE BIOINFORMATICS TOOLS

2.1 INTRODUCTION

Callose is described as a linear β-(1,3)-glucan that occurs ubiquitously in cell walls of intact tissues such as sieve plates, cell plates of newly dividing cells, plasmodesmata, pollen tube development and reproductive organs during sporogenesis and gametogenesis (Northcote *et al*., 1989). It is synthesized rapidly and deposited in a localized manner in response to abiotic stress, wounding, mechanical stress and pathogen attack (Kauss, 1987; Bolwell, 1993; Benhamou, 1995). It is an important component of the responses to environmental stresses that are alleged to occur at the plant cell surfaces and lead to modification of the extracellular matrix (McCormack *et al*., 1997). Callose is said to be electrolucent when viewed in electron micrographs (Stone & Clarke, 1992) and it fluoresces under UV light when stained with aniline blue fluorochrome.

Aphids are known as phloem feeders that are distributed all over the world, resulting in serious yield loss in cultivated plants. Plants have developed chemical and physical defence mechanisms to overcome herbivore attacks. These defence mechanisms are either constitutive or induced by stress (Giordanengo *et al*., 2010). While other studies reported that some aphids prevent callose formation and protein plugging, others have revealed that some aphid species cause callose formation (Botha and Matsiliza, 2004; Kusnierczyk *et al*., 2008). Therefore this study was conducted to evaluate whether bioinformatic tools could be used to predict whether callose synthases are potentially up-regulated during biotic stress and in particular in response to aphid infestation.

2.1.1 CALLOSE DEPOSITION

Callose deposition has been described as a typical plant response to stress aimed at locally isolating the impact of the stress in the plant tissue through the deposition of a physical barrier (Kauss, 1989; Farrokhi *et al*., 2006). The formation of callose deposits in mechanically wounded plant cells in response to disruption of active sieve tubes during specimen preparation was first described by Fischer (1886). Many studies have shown that localized deposits of callose develop at the periphery of a variety of plant cells subjected to

mechanical stress (Aist, 1976; Fincher and Stone, 1981; Kauss, 1985). When tissues are damaged, callose is first deposited to walls of the surviving cells in wound healing according to Galway and McCully (1987). Currier and Webster (1964) showed that different types of callose deposits were synthesized in cotton petioles at different times post wounding and also that these different deposits remained for variable times. Wound callose is first deposited at intercellular connections (i.e. sieve plate pores and pit fields) and spreads to surrounding wall regions (Currier, 1957).

Callose is formed within minutes of wound initiation and is deposited between the plant cell wall and the plasma membrane (Nakashima *et al.,* 2003; Radford *et al*., 1998). The rapid deposition of callose is known as an efficient wound response that seals off the pores in damaged phloem to prevent loss of assimilate (Sjolund, 1997). Locally deposited callose in sieve pores and plasmodesmata could take place either as a response to wounding or as some other physiological stresses, and can also be deposited as plugs or plates (papillae) in response to infection by pathogens (Stone and Clarke, 1992; Donofrio and Delaney, 2001).

Botha & Matsiliza (2004) have reported that Russian wheat aphid (RWA) (*Diuraphis noxia* Mordvilko), known for causing leaf rolling, is also one of the aphids causing callose deposition in cereals. Studies have shown that the infestation of wheat by RWA causes localized callose deposition in wheat (Botha and Matsiliza, 2004). Other studies have revealed that barley leaves infested with RWA showed chlorosis, neurosis, while the barley leaves infested with BCA (Bird-Cherry oath aphid) had no visible damage (Saheed *et al*., 2007; Saheed *et al*., 2009). Larger BCA populations, however cause callose deposition in Barley (Saheed & Botha, 2010). On wheat and other cereals RWA and BCA occur at the same time, with BCA causing no visible damage on the plant (Messina *et al*. 2002). Studies have shown that RWAS1 infestation on cereal, causes severe damage to the plant phloem and xylem transport (Botha and Matsiliza, 2004; de Wet and Botha, 2007; Saheed *et al*., 2007a, 2007b, 2009, 2010). It was further reported by Saheed *et al*., (2007a, 2007b) that RWASA1 causes structural damage on vascular tissues in wheat and barley. Some studies have been done on the interaction between potato aphid known as *Macrosiphum euphorbiae* and the tomato plants *solunum lycopersicum*. Most commercial tomatoes have been reported to contain *Mi-1.2* that confers resistance to potato aphids (Rossi *et al*., 1998; Milligan *et al*., 1998), which leads to callose deposition.

2.1.3 CALLOSE SYNTHASES

Wounding of plant cells and initiation of wound callose formation are a result of elevated calcium ion levels in a mechanism involving calmodulin (Botha and Cross 2001). sucrose synthase (SuSy) exists in a complex with the β-(1,3)-glucan synthase and serves to channel glucose from sucrose to the glucan (Amor *et al*. 1995 and Salnikov *et al*. 2003). In *Arabidopsis thaliana* 12 glucan synthase-like (GSL) genes have been identified. GSL5 has been reported to function in callose deposition after injury and pathogen treatment on plants (Jacobs *et al*., 2003). GSL1 and GSL5 act excessively in the production of a callosic wall that prevents microspore degeneration, and both are required for fertilization (Enns *et al*., 2005).

GSL2 is needed for patterning of the pollen exine in the callosic wall around pollen mother cells, (Dong *et al*., 2005), and also plays a major role in callose deposition in the wall and plugs of pollen tubes (Nishikawa *et al*., 2005). GSL8 and GSL10 are required for the asymmetric division of microspores and for the entry of microspores into mitosis (Toller *et al*., 2008; Huang *et al*., 2009). GSL6 (CalS1) is involved in the cell plate′s callose synthases, since a 35S::GFP-GSL6 fusion in transgenic BY-2 tobacco (*Nicotiana tabacum*) cells increases callose induction, and GFP fluorescence is found only at the cell plate (Hong *et al*., 2001). GSL8 (CalS10) is needed for normal cytokinesis and its mutants exhibit excessive cell proliferation and abnormal cell patterning, however its phenotypes have not been previously reported for cytokinesis defective mutants. CalS1 was identified in the growing cell plate and interacted with two cell plate–associated proteins which are phragmoplastin and a UDP-glucose transferase (Hong *et al*., 2001a, 2001b). *AtGSL6* induces callose deposition at the cell plate, but does not complement the yeast mutant *fks1* (Hong *et al*., 2001a). However reports on *AtGSL5* have shown that, this gene partially complements *fks1* and is induced by salicylic acid (Østergaard *et al*., 2002). GSL5 is required for wound and papillary callose formation. *AtGSL5* is highly expressed in flowers (Østergaard *et al*., 2002).

2.1.4 PROMOTER IDENTIFICATION (software driven process)

The identification of the transcriptional network starts with the identification of the promoter. Transcriptional binding sites (TFBS) are derived from a set of co-regulated genes, or a set of genes that have similar functions but different procedures. Specific binding elements are then combined in one recognition module/model, used in screening of the genome for identifying genes regulated by similar mechanisms. The phylogenetic foot printing (PF) approach is then used to reduce the size of the genomic regions to be searched for target genes. Specific genes contain specific combination of TFBS in their promoter region thus determining the temporal and spatial expression. Signal-based approach uses a neural network and is highly dependent on the recognition of relatively conserved signals (TATA box, CAAT box and TFBS) and conserved spacing among them (promoter 2.0, Jiashun *et al*., 2003; *PromH,* Solovyev and Shahmuradov, 2003). A content-based approach distinguishes promoter sequences from non-promoter ones by comparing nucleotide contents such as triplet base-pair preferences around transcription start sites (TSS), hexamer frequencies in consecutive 100 bp upstream regions. The programs properly predict about 13–54% of the promoters but also include false-positive. However the promoter Inspector system that has been developed produces a reduced level of false-positive recognition (Scherf *et al*., 2000).

2.1.5 *CIS*-REGULATORY ELEMENTS

The diverse cell type formation from an invariant set of genes requires biochemical and molecular biological processes that regulate the activity of the genes. The most studied process in cell and molecular biology is transcription which is the central step in gene expression (Wyeth & Albin, 2004). Transcription is initiated by the interaction between transcription factors that bind the *cis*-regulatory elements in the DNA, additional co-factors and is further influenced by chromatin structure. The trans-acting proteins controlling transcription rate at the level of individual genes bind crucial *cis*-regulatory sequences. Therefore clear knowledge of the interaction between trans/acting factors and *cis*-regulatory sequences would provide a means to interpret and model the responses of cells to diverse stimuli. Computational methods used in identifying *cis*-regulatory sequences associated with genes still requires more laboratory procedures that are used in identifying them (Geffery *et al*., 2007).

2.2 MATERIALS AND METHODS

2.2.1 IDENTIFICATION OF CALLOSE SYNTHASES

Complete nucleotide sequences for callose synthases were obtained from GenBank (www.ncbi.nlm.nih.gov) using the accession numbers reported by Yamaguchi *et al*. (2005) for rice sequences: OsGSL1 (AP001389); OsGSL2 (AP003223); OsGSL3 (AP003268); OsGSL4 (AP003445); OsGSL5 (AP003454); OsGSL6 (AP004685); OsGSL7 (AP004082); OsGSL8 (AC118980); OsGSL9 (AP003447); OsGSL10 (AP0014426) for *Arabidopsis thaliana* sequences: AtGSL1 (NM_116736.1); AtGSL2 (NM_179622.3); AtGSL3 (NM_001202724.1); AtGSL4 (NM_112317.2); AtGSL5 (NM_116593.3); AtGSL6 (NM_100436.4);AtGSL7 (NM_100528.2); AtGSL8 (NM_179940.5); AtGSL9 (NM_123045.3); AtGSL10 (NM_111596.6); AtGSL11 (NM_115772.3); AtGSL12 (NM_121303.7) for Wheat ((*Triticum aestivum L*.) partial cds sequences: *TaGSL2* (DQ086483); *TaGSL3* (DQ086484); *TaGSL8* (DQ086485); *TaGSL10* (DQ086486); *TaGSL12* (DQ086487); *TaGSL19* (DQ086488); *TaGSL22* (DQ086489); *TaGSL23* (DQ086490) and for Barley sequences as reported by Li et al; 2003: BU982241; YA177665; BE55901; BJ484184; BQ484184; BJ4779194.

2.2.1.1 Identification of the 2000 base pair promoter region upstream of the 5′end of the coding region

For rice callose synthases, a RAP-DB rice database (http://rapdb.dna.affrc.go.jp/) was used in the identification of the 2000 base pairs 5′ upstream from the start codon of the coding region of each sequence. For the *Arabidopsis thaliana* callose synthases, the genomic database was searched for the 1500 base pairs 5′ upstream from the start codon of each coding region of each sequence.

2.2.1.2 *CIS*-REGULATORY ELEMENT ANALYSIS

The 5′ upstream regions obtained as described in Section 2.2.1.1. were used in scanning for the presence of putative *cis*-regulatory elements using PLACE (http://www.dna.affrc.go.jp/PLACE/signalscan.html); PLANTCARE (http://bioinformatics.psb.urgent.be/webtools/plantcare/cgi-bin/CallMatIF55.htpl) , all *Arabidopsis thaliana* sequences were converted to genes using the NCBI database and were used to search for Transcription factors in ATHENA (http://www.bioinformatics2.wsu.edu/cgi-bin/Athena/cgi/home.pl) databases. A BLASTN database search at the NCBI database and the rice genome or *Arabidopsis* genome was performed using each

of the rice and *Arabidopsis* 5′ upstream sequences identified in section 2.2.1.1. to ensure that these sequences did not encode any proteins or portion of proteins.

2.2.1.3 Genevestigator Analysis (Software driven process)

Arabidopsis thaliana callose synthase sequences described in section 2.2.1 were used to obtain the gene accession numbers in ncbi database (www.ncbi.nlm.nih.gov). This was done by selecting a nucleotide database and searching for each gene using the accession numbers: AtGSL1 (AT4G04970); AtGSL2 (AT2G13680); AtGSL3 (AT2G31960); AtGSL4 (AT3G14570); AtGSL5 (AT4G03550); AtGSL6 (AT1G05570); AtGSL7 (AT1G06490); AtGSL8 (AT2G36850); AtGSL9 (AT5G36870); AtGSL10 (AT3G07160); AtGSL11 (AT3G59100); AtGSL12 (AT5G13000). The above mentioned gene codes for *Arabidopsis thaliana* were used in searching genevestigator analysis (https://www.genevestigator.com/gv/). A plant biology search was done in the genevestigator analysis database, and each gene code was used for each specific gene search. Samples was selected in the database and searched for aphids information

2.3 RESULTS AND DISCUSSION

Phylogenetic analysis (Figure 2.1) of the ten rice, twelve *Arabidopsis*, eight barley and eight wheat callose synthase genes resulted in 4 subgroups with three of the four groups each containing GSL from rice and *Arabidopsis* thaliana members. This suggests that functional differentiation of plant GSL occurred before monocot-dicot divergence and that there is possible degeneracy amongst the various GSLs.

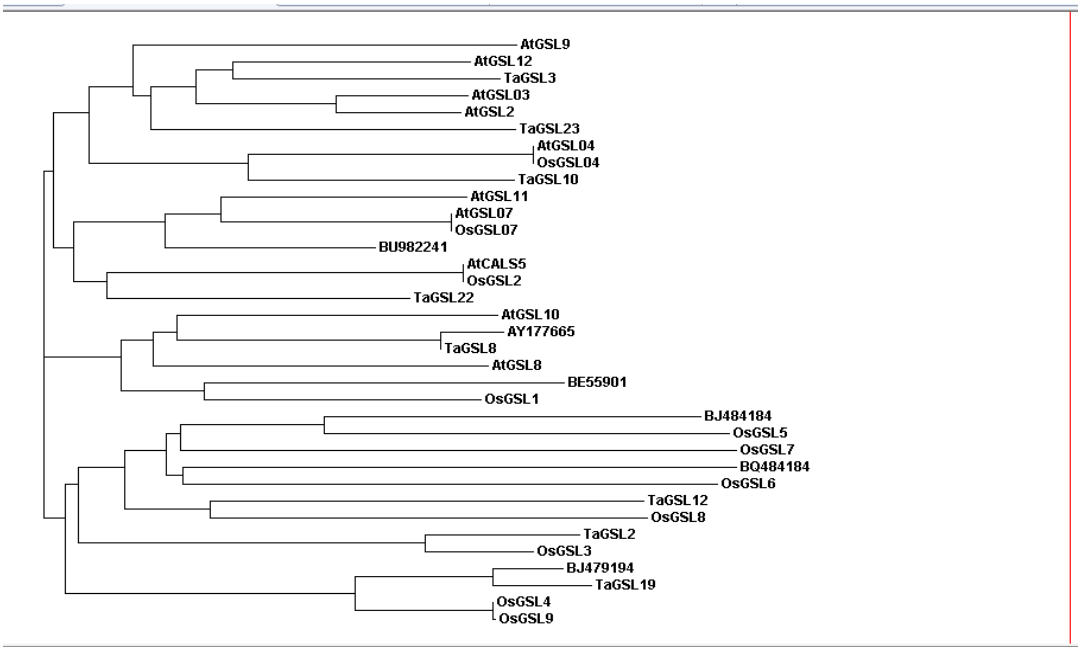


Figure 2.1: Phylogenetic tree based upon the alignment of full length mRNA coding sequences of *Arabidopsis* thaliana, rice callose, barley and partial mRNA wheat callose synthases. Callose synthase sequences used in creating this phylogenetic tree were obtained from GenBank (www.ncbi.nlm.nih.gov) using the following sequences OsGSL1 (AP001389); OsGSL2 (AP003223); OsGSL3 (AP003268); OsGSL4 (AP003445); OsGSL5 (AP003454); OsGSL6 (AP004685); OsGSL7 (AP004082); OsGSL8 (AC118980); OsGSL9 (AP003447);OsGSL10 (AP0014426) and for *Arabidopsis thaliana* sequences: AtGSL1 (NM_116736.1); AtGSL2 (NM_179622.3) ; AtGSL3 (NM_001202724.1) ; AtGSL4 (NM_112317.2); AtGSL5 (NM_116593.3); AtGSL6 (NM_100436.4); AtGSL7 (NM_100528.2); AtGSL8 (NM_179940.5); AtGSL9 (NM_123045.3); AtGSL10 (NM_111596.6); AtGSL11 (NM_115772.3); AtGSL12 (NM_121303.7).

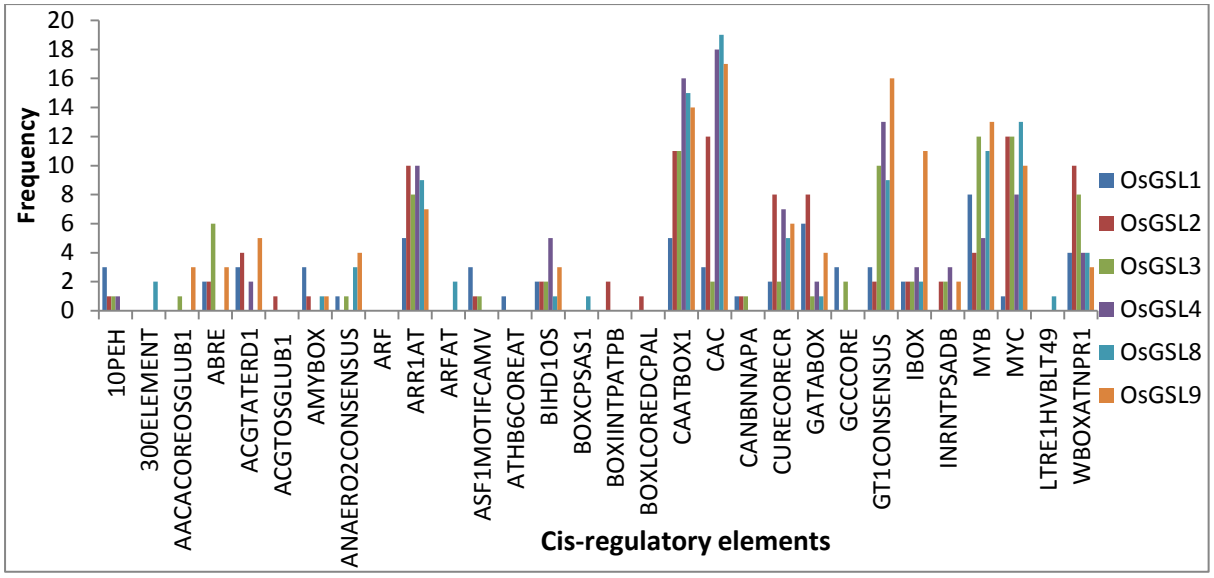


Figure 2.2: A graphical illustration of *Oryza sativa cis* regulatory elements identified in the 2000bp 5′ upstream regulatory sequences of the callose synthases.

The *cis* elements identified for the rice callose synthases indicated that they are mainly regulated by abiotic stress. The *cis* regulatory elements identified were involved in development, drought, light and extreme temperature responses with only one *cis* element (W-box) linked to wounding or pathogen infection. This supports the evidence of callose deposition in conditions such as drought or high salinity and a possible role for all the rice GSLs playing a role in wounding.

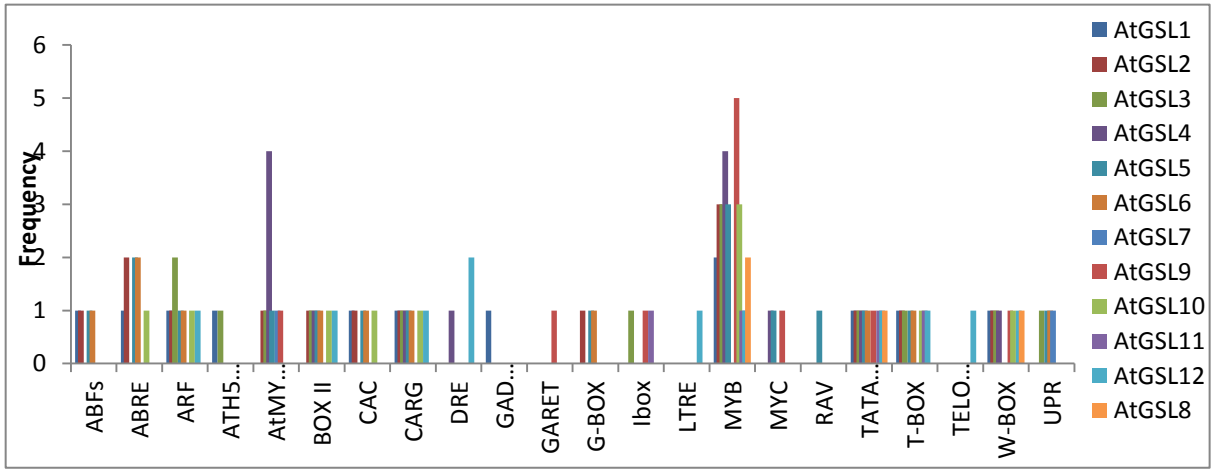
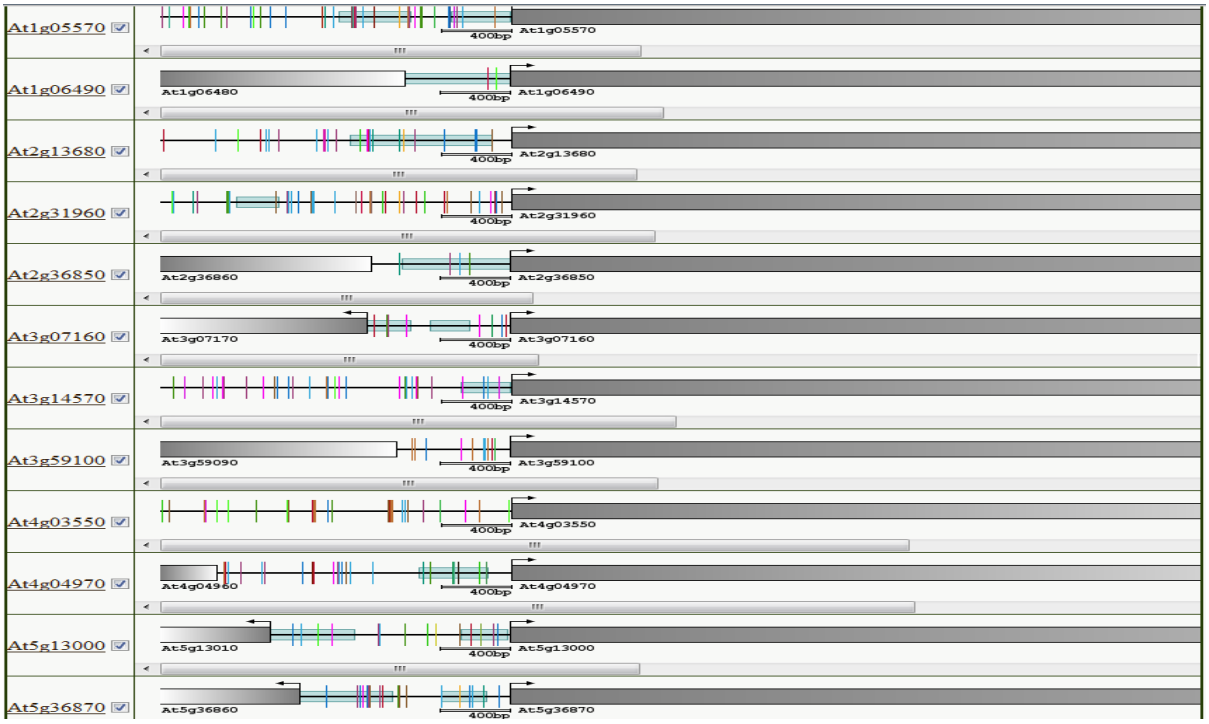


Figure 2.3: A graphical illustration of *Arabidopsis thaliana cis* regulatory elements identified in the 2000bp 5′ upstream regulatory sequences of the callose synthases.

(A)



(B)

Figure 2.4: (A) Compact visualization of *cis* elements for in the 1500 bp 5′ upstream region of the twelve *Arabidopsis thaliana* callose synthase genes. Each TF non-binding site is indicated by a different colour that matches the colour in the TF table. An arrow

represents the transcription start site and the dark grey rectangle the predicted coding sequence. The light blue small rectangles represent the CpG islands. (B) Non-Enriched TF binding sites present in the 1500bp 5′ upstream promoter region of *Arabidopsis thaliana* callose synthase genes.

Table 2.1: Genevestigator analysis results of Arabidopsis thaliana callose synthases in aphids

Gene	GEO accession code	Signal value	p-Value	Expression level	Signal log2 value
AtGSL1	GSE6172	2560,3323,3197,3014,2904	<0.01	Medium	11.32,11.70,11.64,11.56,11.50
AtGSL2	GSE16497	315,356,343,342,299,290	0.32,0.29, 0.39,0.26, 0.83,0.81	Low	9.12,9.03,9.12,9.12, 9.17
AtGSL3	GSE16497	480,3429,3204, 3820,3842, 3873	<0.01	Medium	12.00,12.77,11.99,12.30,12.13,
AtGSL4	GSE16497	390, 435, 421,335, 310,354	0.01,0.03, 0.02, 0.32, 0.14	Low	9.35,9.449.64,9.41,9.44
AtGSL5	GSE16947	2746, 2731,3386,2324,2639,3220	<0.01	Medium	11.92,11.9211.99,11.8811.92
AtGSL6	GSE16947	4277,3772,3618,3546,3546,4362	<0.01	Medium	,11.7911.98,12.07,12.0412.45
AtGSL7	GSE6172	1208,1104,1051,1181,1181	0.01,0.03,0.02	Medium	10.24,10.11,10.0410.21,10.21,
AtGSL8	GSE6172	3096,3194,311431953312	<0.01	Medium	11.6011.64,11.60,11.64,11.69,
AtGSL9	GSE6172	731,731,698,733,731	0.03,0.12,0.06,	Low	9.51,9.51,9.45,9.52,9.51
AtGSL10	GSE6172	5754,56065710,5766,5810	<0.01	High	12.49,12.53,12.48,.12.49,12.
AtGSL111	GSE6172	2031,1925,1891,1804,1995	0.01, <0.01	Medium	10.99, 10.91, 10.89, 10.82, 10.96
AtGSL12	GSE6172	5828,5114,5799,4667,5042	<0.01	Medium	12.51,12.32,12,50,12.19,12,30

Twelve *Arabidopsis thaliana* callose synthase sequences were obtained from GenBank (www.ncbi.nlm.nih.gov). Twenty two *cis* regulatory elements were identified by Athena, most involved in transcription regulation during abiotic stresses with only one, (W-box) involved in the wounding response and pathogen infection.

Genome sequences for multiple species and large-scale gene expression data availability gave rise to the development of computational genomic approaches that are used in studying transcriptional regulation. However, this field is challenged by accurate genome-wide identification of the regulatory targets of transcription factors (TF), which is the key step towards reconstructing cellular transcriptional networks. A lot of functional genomic approaches have been developed to attack this problem. ChIP-chip (chromatin immunoprecipitation followed by hybridization to DNA chip) technology has been used on a large scale in mapping the location of transcription factors in the yeast genome (Agrawal and Sheriff 2001; Amor *et al*., 1995). Gene expression profiling of cells in which a transcription factor is either overexpressed or deleted has also been used to identify targets (Botha and Matsiliza, 2004; De Wet *et al*., 2008).

As discussed in section 2.1.1 above, callose deposition has been reported in cereals in response to RWA infestation. However, studies looking at transcriptional regulation of barley callose synthases showed no difference in the transcription levels of callose synthases between (Saheed *et al*., 2009) infected and control plants. This study did not include all barley GSL genes in the microarray and it is therefore possible that other barley callose synthase encoding genes could be involved in the response to aphid infestation.

Based on the results of the identification of *cis*-elements regulating the callose synthases in rice and *Arabidopsis*, it would appear that callose synthase expression is generally regulated by abiotic stress or plant development due to the presence of *cis*-elements such as MYB, DRE, ABRE, TATA BOX, and CAAT BOX. The presence of a single *cis*-element (W-box - which is related to wounding responses in plants), in the promoter region of all the rice and *Arabidopsis* callose synthases analysed, could indicate a possible link between transcriptional regulation of callose synthase and aphid infestation but this is unlikely since no other *cis*-elements related to biotic stress or pathogen infection were identified. Genevestigator analysis of microarray studies involving aphid infestation of *Arabidopsis* indicated that AtGSL10 is highly up-regulated, thereby showing a strong implication for a role in the *Arabidopsis* response to aphid infestation. AtGSL2, AtGSL4 and AtGSL9 showed no up-

regulation and therefore are not involved in the plant response to aphid, whereas the remaining AtGSL genes (1, 3, 5, 6, 7, 8, 11 and 12) were shown to have a medium up-regulation, therefore indicating a possible involvement with *Arabidopsis* response to aphids.

In conclusion, analysis of the promoter region of callose synthases using available bioinformatics tools was not able to definitively confirm whether callose synthases are expressed in response to biotic stresses of aphid infestation, whereas microarray data from Genevestigator indicated that AtGSL10 is involved in the *Arabidopsis* response to aphid infestation. The *cis* analysis approach is also limited to the availability of genome sequence data and therefore it is not possible to confirm whether all callose synthases are not regulated by aphid infestation (GSL5, 6 and 7 sequencing was still in progress at the time of this study) nor can this approach determine the existence of a species specific response unless genome sequences are available for all species. Wet bench research is therefore still the preferred approach of choice to identify proteins involved in the stress or resistance response of cereals to aphid infestation, indicating the need for future proteomic studies.

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CHAPTER 3

ANALYSIS OF WHEAT PROTEOME AND PHOSPHOPROTEOME USING LC/MS

3.1 INTRODUCTION

Proteomics tools are used to determine protein structures, their function and total protein expression in a given system (Nakamura & Oda, 2007). Mass spectrometry has become an invaluable tool in proteomics to identify differentially regulated proteins as well as quantitate proteins in high throughput systems (Aebersold & Mann 2003; Domon & Aebersold, 2006; Elias *et al*., 2005). Liquid chromatography mass spectrometry (LC/MS) has become popular over the last few years as the sensitivity and accuracy of the systems have increased (Ong and Mann, 2005;Wilm, 2009). A major limiting factor in the LC/MS-based quantification via electrospray is the suppression of ions (Tang *et al*., 2004). Thus the intensity of peptides is highly dependent on the quality of peptides that are ionized, ionisation efficiency and the properties of peptides being eluted (Tang *et al*., 2004, Schmidt *et al*., 2003, Annesley *et al*., 2003,Elliot *et al*., 2009). Other limitations that LC/MS-based approach is facing are separation peak capacity, reproducibility of the chromatography and mass measurement accuracy and the resolving power of mass spectrometry. The development of ultra-performance LC and high-mass accuracy/resolution mass spectrometry have solved many of these problems, thus LC/MS-based quantification has become more reliable and easy to access to biologists (Xie *et al*., 2011). The stable isotope labelling methods are used in proteomics by combining samples after labelling followed by analysis by LC-MS, thereby avoiding the uncertainty induced by variations in instrument performance between measurements. However, proteomics labeling techniques have limitations which has lead to a growing interest in quantitative "label free" analyses together with low flow rate separations. (Callister *et al*., 2006).

3.2 MATERIALS and Methods

3.2.1 PLANT MATERIAL CULTIVATION

3.1Two wheat strains, Tugela (susceptible to aphid strain) and Tugela DN (aphid resistant strain) were used in this study. Wheat seeds were germinated as described by Scofield *et al*. (2007a). Seeds were rinsed twice in 1% bleach followed by 70% ethanol, before being placed on sterile, moist Whitman filter paper in a petri dish.

Susceptible and unsusceptible wheat genotypes plants were grown under controlled environmental conditions in a Conviron S1OH incubator/growth chamber manufactured by Controlled Environments Ltd., Winnipeg, Manitoba, Canada, under the following conditions: temperature of 25^{o}C, 56% humidity, CO_2 (ambient) and a light intensity of 400 µmol $m^{-1}sec^{-1}$ and a day/ night cycle of 16hr/ 8 hr. A total of 48 plants (24 Tugela DN and 24Tugela) were used with 2 plants per pot per time slot for control (with no aphids) and for test (with aphids) for both Tugela DN and Tugela strains. These plants were allowed to grow to a two leaf stage, before infestation with the Russian wheat aphids (RWA). Approximately ten aphids were applied to a leaf of each plant within a leaf cage. Control plants with no aphids still had the leaf cage applied. Aphids were allowed to feed on the leaves for nine days, with leaves harvested at various times: Day 0 (0 hrs), Day 1 (24 hrs), Day 3 (72 hrs), Day 5 (120 hrs), Day 7 (168 hrs) and Day 9 (216 hrs) post infestation. Control plants were treated the same as test pots, except for the application of aphids on the control plants. All aphids were brushed off on harvesting to make sure that no aphids were left on the leaf, and leaves were immediately frozen in liquid nitrogen and stored in a -80 ºC freezer until further processed.

3.2.2 TOTAL PROTEIN EXTRACTION

All equipment needed for the extraction of protein was sterilized by autoclaving. Total protein was extracted from the harvested leaves using ReadyPrep™ Protein extraction kit (total protein) (Bio-Rad, US) following the manufacturer′s instructions. In summary, collected wheat leaves were frozen in liquid nitrogen and using sterile pestle and mortar were ground into a fine powder and quickly transferred into a sterile 15 ml round bottom

falcontube and weighed. Per 500 mg of powdered sample, 2.0 ml of 2-D rehydration/sample buffer was added. Samples were then placed on ice and the suspensions were sonicated with an ultrasonic probe to disrupt the cells and fragments of genomic DNA. Samples were sonicated using 3 times 30 seconds bursts. Suspensions were chilled briefly on ice between each sonication treatment. The extract was then transferred to 2 ml microcentrifuge tubes. Tubes were centrifuged using a benchtop centrifuge at a maximum speed of 16000 x *g* for 20 minutes at room temperature to pellet the cell debris. The supernatants were transferred into sterile 2 ml microcentrifuge clean tubes and the remaining insoluble pellet discarded. Samples were stored at -80ºC until required.

3.2.3 MICRO BCA PROTEIN ASSAY

A modified micro BCA protein assay kit protocol (Thermo Scientific) was used in determining the protein concentrations following the manufacturer′s instructions. The proteins standards were prepared by diluting BSA standard (2.0 mg/ml) provided with the kit into several clean vials with rehydration/sample buffer used above (protein extraction). The reagent working solution was prepared by mixing 12.5 ml of reagent MA, 12.0 ml of reagent MB with 0.5 ml of reagent MC. In a microplate well, 150 µl of the working solution was added to 150 µl of sample/standard (in triplicate) and mixed thoroughly on a shaker for 30 seconds. The plate was covered using parafilm and incubated at 37ºC for 2 hours. The plate was cooled to room temperature and the absorbance was measured at 562 nm. The standard curve was prepared by plotting the average blank-corrected 562 nm reading from each BSA standard vs. concentration in µg/ml (Figure 3.1); the standard curve was then used to determine the concentration of the unknown samples.

3.2.4 SDS-PAGE

Proteins extracted from Tugela and Tugela DN (Section 3.2.2) were prepared of SDS-PAGE gel electrophoresis by adding 6µl of sample buffer (1.25 ml 0.5 M Tris-HCl pH 6.8, 2.5 ml glycerol, 2.0 ml 10% (w/v) SDS, 0.2 ml 0.5% (w/v) bromophenol blue, 3.55 ml deionized water, 50µl β-mercaptoethanol). Samples were then incubated @ 99°C in a master Cycler for 4 minutes and centrifuged @ 13,000 rpm for 1 minute. Prepared proteins were separated by SDS-PAGE and 25 µl of each sample was loaded per lane. SDS-PAGE was carried out using a 12% resolving gel [40% acrylamide (6 ml), 1.5M Tris-HCl pH 8.8 (5 ml), 10% SDS (200

µl), deionized water (8.6 ml), 10% Ammonium persulphate 200 µl, TEMED (8 µl)] with a 4% stacking gel [40% acrylamide (0.2 ml), 1M Tris-HCl pH 6.8 (0.35 ml), 10% SDS (20 µl), deionized water (1.505 ml), 10% Ammonium persulphate (20 µl), TEMED (20 µl)] in a 1X Tris/HCl running buffer system. Electrophoresis was carried out in a Bio-RAD Mini Protein II System at a constant voltage of 200 V for 45 minutes. After separation was complete,gels were washed in 200 ml distilled water with gentle agitation for 5 minutes and then fixed in methanol/acetic acid/water (30/10/60), stained with Coomassie Brillient Blue R250 (Bio-Rad, US) for 2 hrs following the manufacturer′s instructions and destained overnight in a fresh methanol/acetic acid/water (30/10/60) destaining solution. Gels were then visualized and photographed using Alliance 4.7 Transilluminator (UVITEC Ltd, Cambridge, UK).

3.2.5 PROTEIN DIGESTION AND IDENTIFICATION BY NANOLC-MS/MS

Formic acid (FA), Acetonitrile (ACN), Ammonium bicarbonate, Dithiothreitol (DTT) and Iododacetamide (Sigma Aldrich) and Trypsin (Promega,20 µg / vial seq grade modified) were used. Solution preparation volumes were depended on the number of gel slices. Fifty milliliters of 0.1M NH_4HCO_3 was prepared by adding 0.4 g of NH_4HCO_3 to 50 ml of distilled water. Ten milliliters of 10 Mm DTT was prepared by mixing 0.015g of DTT in 0.1 M NH_4HCO_3. One milliliter of 55 M Iododacetamide was prepared by mixing 0.01 g Iododacetamide with 0.1 M NH_4HCO_3. Trypsin solution was prepared by adding 40 µl of the supplied solution to a vial containing lypholysed trypsin to give a final concentration of 500 ng/µl. For the digest solution 100 µl of 100 mM NH_4HCO_3 was added to 2 µl dissolved trypsin.

(I) Ex*ci*sion of the protein bands from polyacrylamide gels

The gel slabs were rinsed in 200 ml deionized water for 10 minutes with gentle agitation. After 10 minutes the water was decanted and replaced with 200 ml fresh water. The gel slabs were then divided into 5 segments per lane, with RuBisCO being cut out. The ex*ci*sed segments were cut roughly into 1 mm cubes and transferred to a clean 1.5 ml tube (low binding).

(II) Washing of gel pieces

Gel pieces were washed with 5 times gel volumes of water for 15 minutes (approximately 800 µl) with centrifugation. The water from the final wash was discarded and gel pieces were then washed with 800 µl of 50% acetonitrile for 15 minutes. Acetonitrile was discarded and the gel pieces washed with water, followed with 50% acetonitrile and finally 200 µl of 100% acetonitrile. Once the gel pieces became white and stuck together, the acetonotrile was removed with a pipette tip, and the gels dried in a speed vac.

(III) Digestion

The gel pieces were rehydrated on ice in enough trypsin digestion solution to cover the gel pieces at a ~20:1 ratio (100 µl) and incubated at 37ºC for 1 hr or overnight @ ambient temperature

(IV) Extraction of the peptides from gel

After overnight in-gel trypsin digestion of the protein samples, the digest solutions were transferred into a clean 1.5 ml tube. The remaining gel pieces were further rehydrated in 150 µl 5% formic acid, vortexed for 10 minutes and incubated for 15 miniutes at room temperature. The supernatant were obtained by centrifugation for five minutes, the supernatant obtained in this step was added to the supernatant obtained in the overnight trypsin digestion. The above wash step was repeated twice. To the remaining gel pieces, 150 µl of 100% acentronitrile was added, vortexed, and the mixture was incubated for 5 minutes at room temperature. Supernatants were obtained by centrifugation for 5 minutes, and were combined with other supernatants obtained above. These solutions were all dried in the speed vac, to dryness. Peptites were redissolved in 20 µl of 5% formic acid and were therefore ready for LC-MS/MS.

3.2.6 Identification of peptides by LC-MS/MS

Trypsin digested peptides were analysed by nanoLC-MS/MS (Biosystems QTRAP 4000 hybrid ion-trap mass spectrometer fitted with an Agilent 1200 Nanoflow High Performance Liquid Chromatography system). For protein identification 30 µg protein was loaded into the LC-MS/MS. Peptides (4 µl) were loaded in 0.1% formic acid onto a 300 µm ID×5 mm C18 PepMap trap column (Dionex) at a flow rate of 25 µl/min. These peptides were then eluted from the trap column using 0.1% formic acid in acetonitrile on a 75µm ID×15cm C18

PepMap 100 3 μm nanocolumn at a flow rate of 200nl/min. PicoTip emitter (20 μm ID, 10 μm tip ID, New Objective Inc., Woburn, MA, USA) was used to spray samples into a mass spec, with a spray voltage of 1.8 kV. The mass spectrometer was operated in data-dependent acquisition mode with Xcalibur software (Thermo Fisher Scientific). Full scan mass spectra were obtained by the Orbitrap at 150–2000 m/z with a resolution of 30,000. The three most intense ions were determined at a threshold above the 1000 ion trap at normalized collision energy of 35% after accumulation to a target value of 1000. Dynamic exclusion was employed within 30 s to prevent repetitive selection of the peptides (Kong *et al*., 2010).

The data was taken from the ProteoIQ that is used for analysis and filtered twice into normalised spectral counts and actual average spectral counts for each protein sample. Proteins were labelled manulally based to where proteins played a role. In Tugela DN a change in the spectral count was observed either because the DN gene was absent or present, Infested Tugela DN if proteins are up only in the infested DN plants. The MS/MS data was searched agains the Graemene database that contains all sequence data from the Poaceae/cereals resulting in many of them displayed in duplicates.

3.3 RESULTS AND DISCUSSIONS

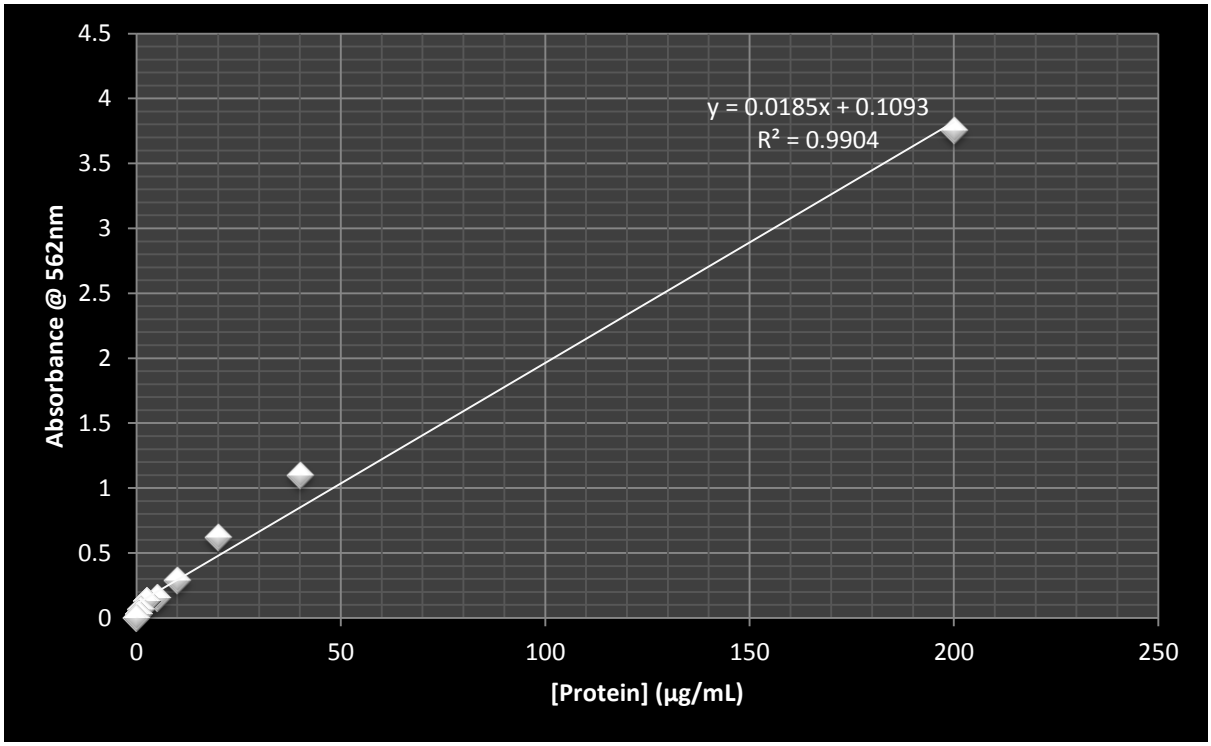

Figure 3.1: Protein standard curve using the micro BCA assay for the protein concentration range 0-250 µg/ml.

Plants were grouped into four groups, Tugela (control/ uinfested); Tugela (infested with aphids); Tugela DN (control/ unifested); Tugela DN (infested with aphids). Each group was made up of five pots containing 2/3 plants per pot. Plants were infested by placing roughly 10 RWA aphids into a leaf cage, while it was attached to one of the four mature leaves per plant.

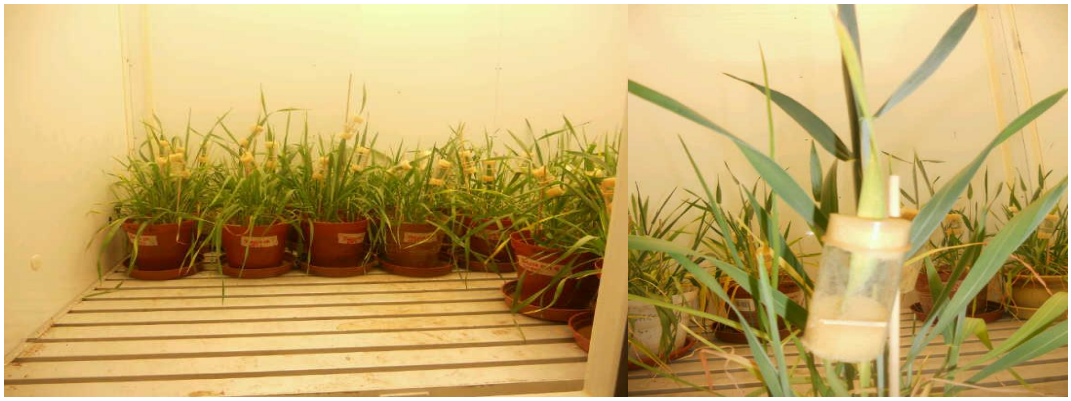

Figure 3.2: A Conviron set up of the Tugela DN and the Tugela wheat plants under controlled environmental conditions under the following settings: temperature of 25^{o}C,

56% humidity, CO_2 (ambient) and a light intensity of 400 µmol $m^{-1}sec^{-1}$ and a day/night cycle of 16 hr/ 8 hr. a) seeds were germinated in petri dishes containing most filter paper. Germinated seeds were transferred into pots that contain soil and placed into a Conviron. Plants were grown to the four leaf stage before being infested with RWA in the setup shown above by placing approximately 10 aphids into a leaf cage attached to one of the leaves. Leaves were harvested at 0, 3, 5, 7 and 9 days and placed immediately into liquid nitrogen.

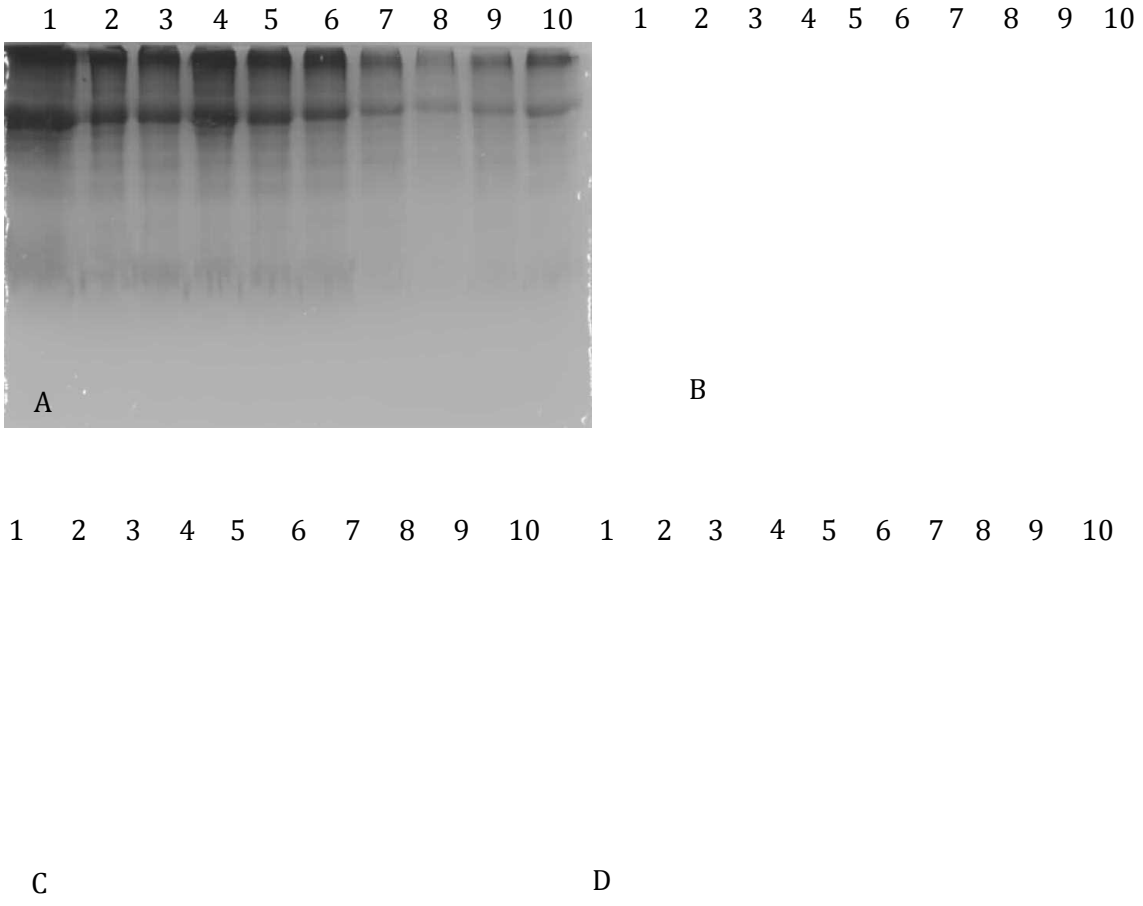

Figure 3.3: 12% SDS-PAGE gels of total protein extracts from wheat infested with RWA. Control plants were treated in the same way as plant infested with aphid, except for the fact that they were not exposed to aphids. Frozen leaves were ground under N_2 in a mortar and pestle, before extraction of total proteins. The integrity was evaluated in 12% SDS-PAGE gels stained with Coomassie Brilliant Blue R-250 (Bio-Rad) showing total soluble proteins extracted from aphid (RWA) infested wheat leaves (Tugela, and Tugela DN) with lanes 1-10 containing soluble proteins that were extracted from susceptible Tugela at 0-216 hours of aphid infestation (A) and uninfested control (B) and resistant Tugela DN (C) and uninfested control (D). No degradation of proteins was observed in any of the samples.

Each lane was divided into five sections and gel pieces cut into small fragments and placed into separate tubes. Gels pieces were washed in a total of 5 washes in 50% acetonitrile and were shrunk in 100% acetronitrile. Addition of 100% acetronitrile resulted in white gel pieces that stuck together. The remaining acetronitrile was then extracted from the gel pieces and the digested peptides were ex*cis*ed from the gel particles by the addition of 5% Formic acid, followed by acetonitrile, and the mixture was centrifuged and incubated at room temperature. The resultant supernatant contained trypsin digested peptides. Digested samples from T0 and T216 (9 days) were subjected to nanoLC-MS/MS for analysis

Differentially expressed wheat proteins identified by nanoLC-MS/MS as stress response related to aphid infestation

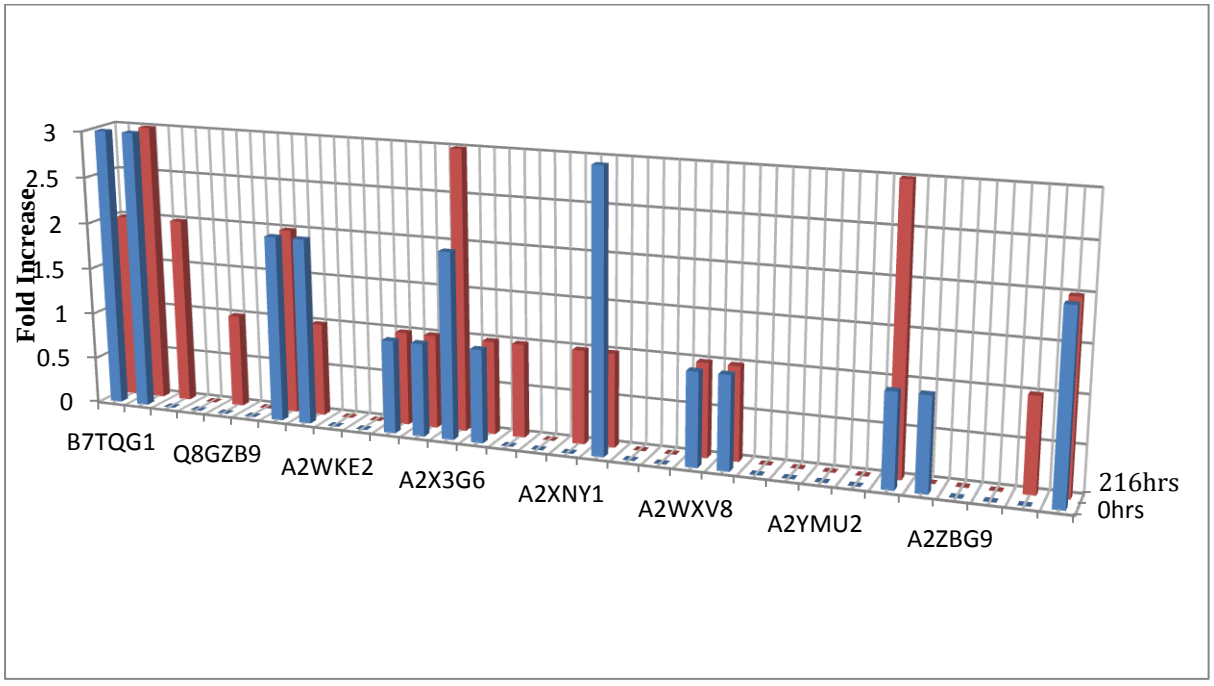

Figure 3.4: A graphical representation of differentially expressed proteins identified to be involved in wheat stress response to aphid infestation. These proteins were either up-regulated or down-regulated when wheat plants were infested with RWA for 216 hrs. The first column represents the Tugela DN stressed protein, 2nd Tugela DN (control), 3rd Tugela (test) and 4th Tugela (control) per accession number. Putative mitochondrial cystein synthase (B7TQG1), putative ascorbate peroxidase (Q8GZB9), putative uncharacterized proteins (A2WKE2, A2X3G6, A2YMU2, A2XNY1, A2ZGB9), fructokinase (A2WXV8), ribosome-recycling factor (A2ZBG9) were all identified as aphid stress responsive proteins.

Pathways identified in stress related wheat proteins during aphid infestation

Cystein synthase pathway

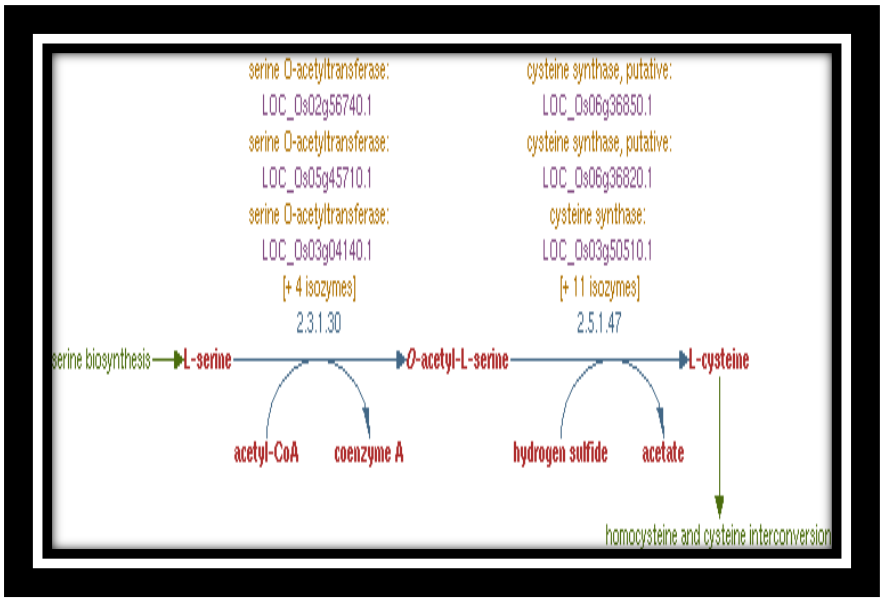

Figure 3.5: The involvement of putative cysteine synthase in cysteine biosynthesis pathway in *Solunum lycopersicum, Oryza sativa* and *Zea maize* (taken from MetaCyc: www.**metacyc**.org; Caspi *et al.*, 2012).

Net Reaction Equation: L-serine + acetyl CoA + sulfide = L-cysteine + CoA + acetate

This pathway of cysteine biosynthesis is a two-step conversion of L-serine to L-cysteine via *O*-acetyl-L-serine. The pathway has been documented in bacteria (Kredich, 1996), archaea (Kitabatake, 2000) and plants (Jost, 2000). Cysteine synthase is a key enzyme in this pathway. Cysteine is one of the compounds that are involved in plant defence systems against toxic heavy metals. Therefore, its synthesis is vital in plant defence.Yang *et al*. (2007) reported up-regulation of cysteine synthase in elavated concentrations of alumium in rice. This enzyme is involved in glutathionine metabolism pathway. Therefore, since this enzyme has only been associated with abiotic stress, and not the wounding stress. The results in this study indicated that cysteine synthase is involved in the wounding stress response, thus futher investigation needs to be done with regards to the association of this enzyme with biotic stresses.

Fructokinase involved in wheat-aphid infestation

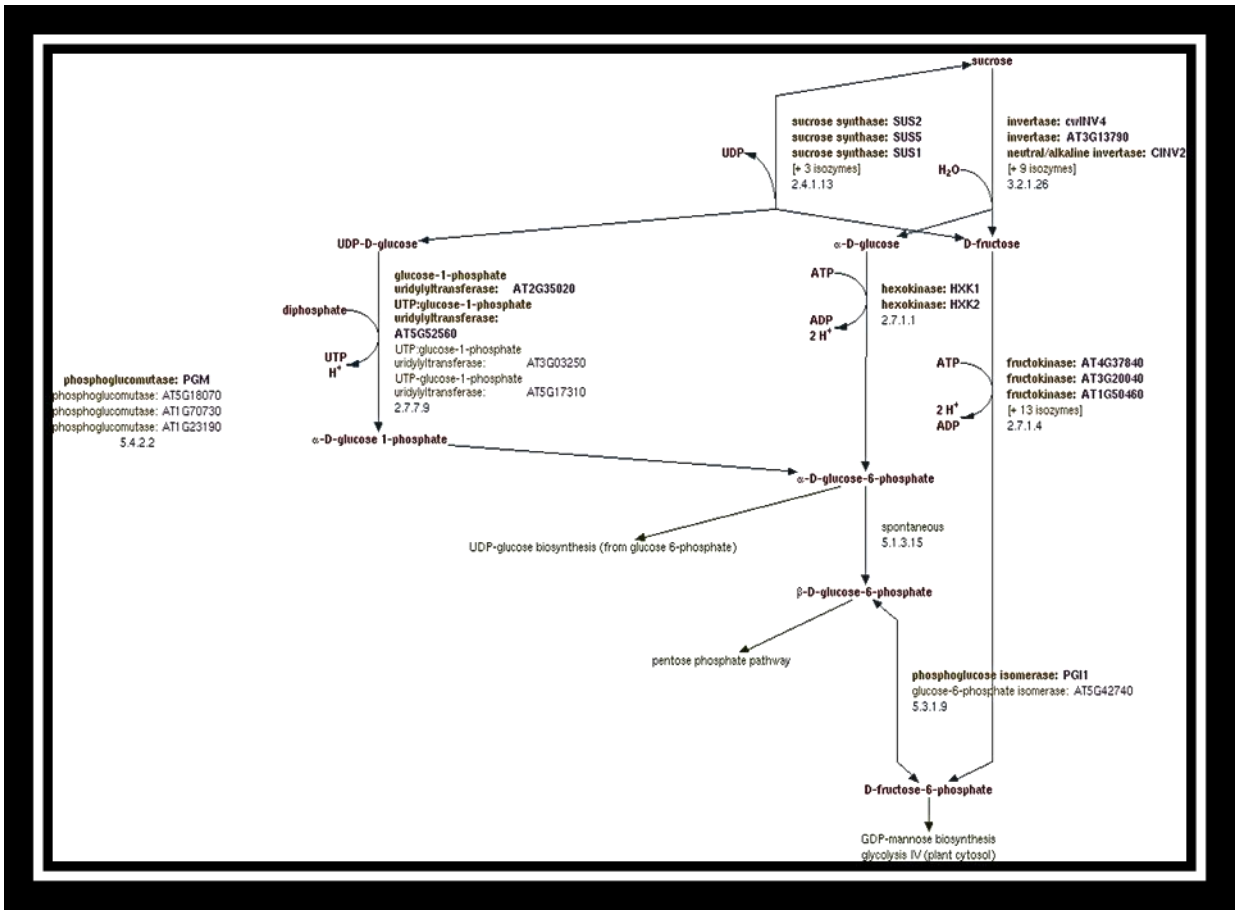

Figure 3.6: The involvement of fructokinase in D-fructose-6-phosphate formation and GDP-mannose biosynthesis (taken from MetaCyc: www.**metacyc**.org; Caspi *et al.,* 2012).

Upregulation of fructokinase has been indicated in both abiotic and biotic stress reponses. Fructokinase which is involved in metabolism has been reported to be downregulated by osmotic stress. Fructokinase plays a major role in sucrose and fructose metabolism. Together with sucrose synthase, fructoskinase is involved in the regulation of starch synthesis in sink plant tissues such as potatoes. A study by Hayashi *et al*. (1998) has reported the upregulation of fructokinase in anther as a result of cold stress. It was further reported that fructokinase is up-regulated in Arabidopsis, under alternating gravity conditions. Fructokinase is up-regulated during fructose phosphorylation, which is associated with the decrease in pool size of fructose. This enzyme is involved in sugar catabolic pathways and in the reduction in cell wall polysaccharides content of roots and more growth under osmotic pressure stress. Shoresh and Gray (2008) showed the up-regulation of fructokinase 2 in maize seedling infected with *Trichoderma harzianum*. In tomato leaves, it has been reported as having a specific role in contributing to stem and root growth, while making plants much shorter (Odanaka *et al*., 2002). It is proposed that its role in aphid wounding stress is related to its role in osmotic stress responses as well as providing strong evidence that, OsSUT1 upregulation in rice plants in response to aphid infestations has a specific function in recovery of sucrose leaked into the xylem apoplast (Ibraheem *et al*., 2013).

Resistance related wheat proteins to aid infestation identified by nanoLC-MS/MS

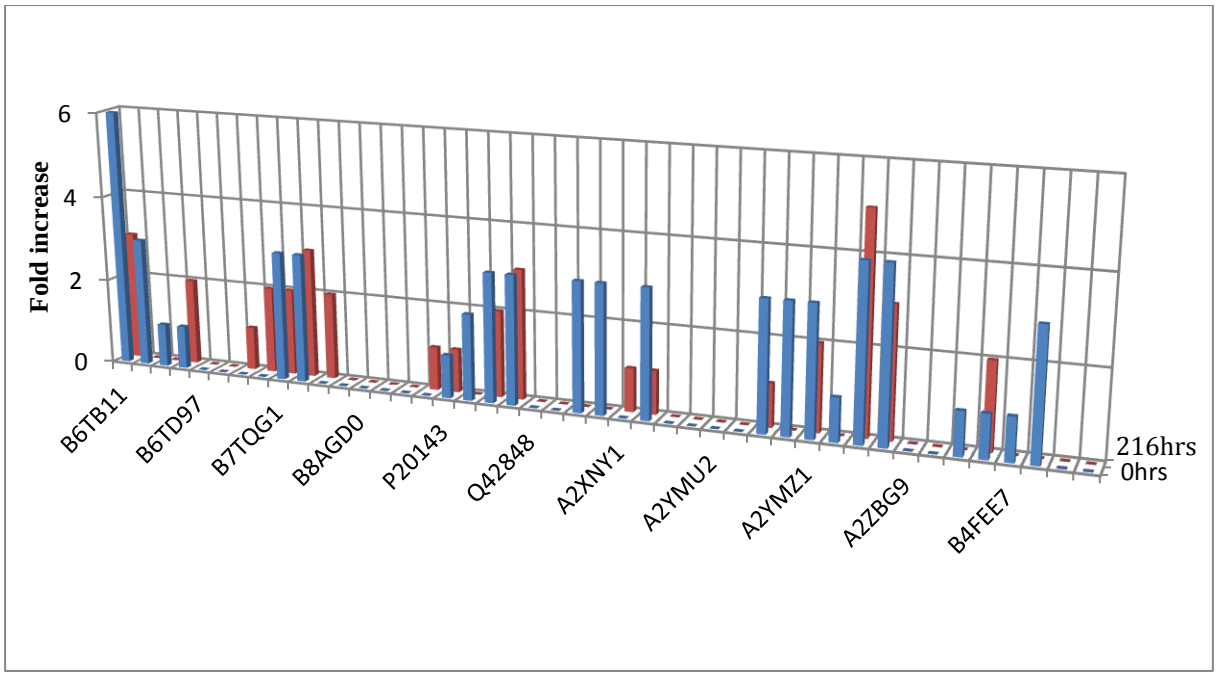

Figure 3.7: A graphical representation of the differentially expressed protein identified to be induced in the Tugela DN resistant mechanism. These are the proteins that only appear in Tugela DN and not in Tugela after the aphid infestation for 216 hrs. The first column represents the Tugela DN stressed protein, 2nd Tugela DN (control), 3rd Tugela (test) and 4th Tugela (control) per accession number. Proteins that were identified as being involved in the resistant mechanism were aldehyde dehydrogenase family 7 members A1 (B6TB11), putative uncharacterized protein (B6TD97), and putative mitochondrial cysteine synthase (B7TQG1), putative uncharacterized protein (B8AGD0), Photosystem I reaction center subunit VI, chloroplastic (P20143), Ribulose bisphosphate carboxylase/oxygenase activase B, chloroplastic (Q42450), putative uncharacterised protein (A2XNY1), Ribosome-recycling factor, chloroplastic (A2YMU2), putative uncharacterized protein (A2YMZ1),putative uncharacterized protein (A2YMZ1), 40S ribosomal protein S26 (B4FEE7).

Pathways identified in resistance related wheat proteins during aphid infestation:

Aldehyde dehydrogenase involved in resistance related wheat-aphid infestations

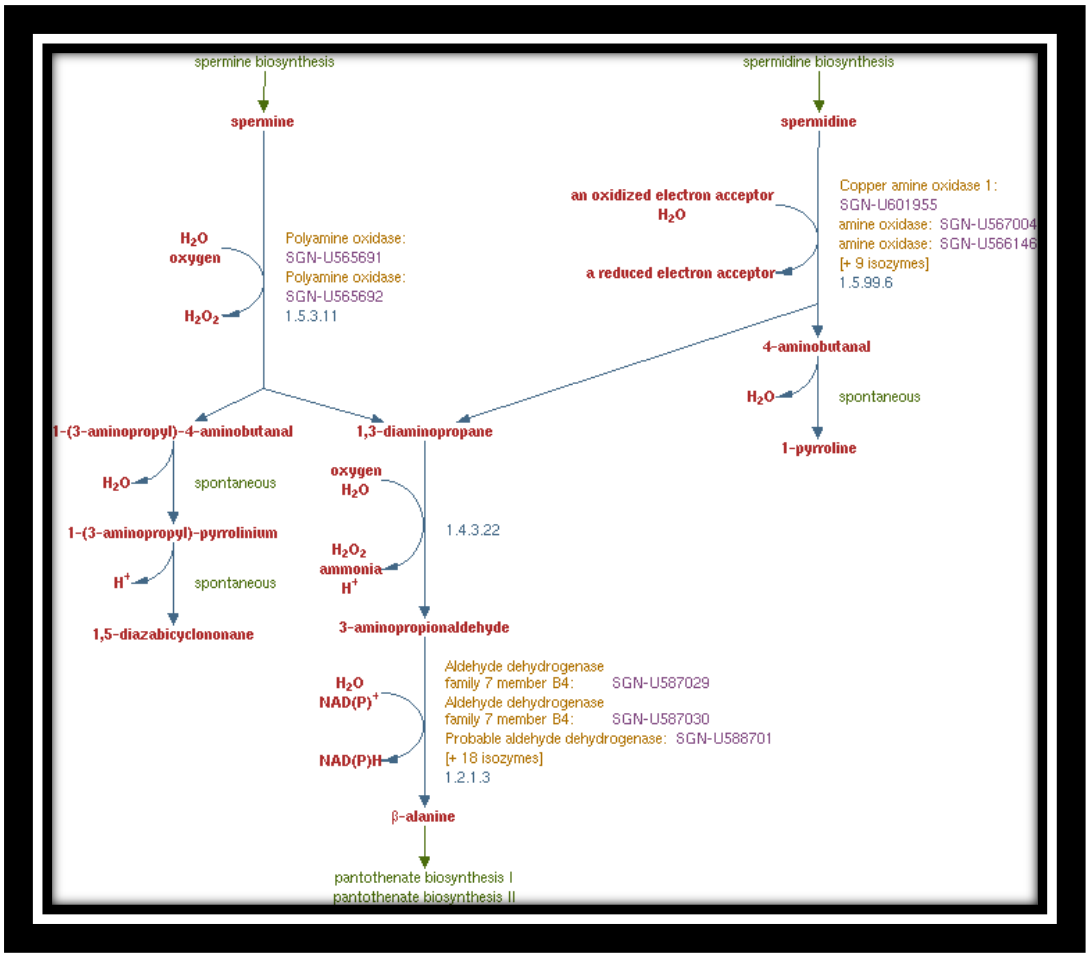

Figure 3.8: Solunum llycopersicum pathway: beta-alanine biosynthesis I. β- alanine is known as the central component of pantothenate biosynthesis (taken from MetaCyc: www.**metacyc**.org; Caspi *et al.*, 2012)**.**

Aldehyde dehydrogenase is associated with the conversion of ethanol to acetaldehyde by cytosolic alcohol dehydrogenase in the ethanol degradation pathway. The resulting acetaldehyde passes into the mitochondrial compartment and is converted to acetate (by mitochondrial aldehyde dehydrogenase). In case acetate is activated to acetyl-CoA within the liver, it is then oxidized by the Krebs cycle because of the prevailing high ratio of $NADH^{+}H$ / NAD^{+} within the liver mitochondrial matrix. Studies have demonstrated oxidative and nonoxidative pathways in various tissues such as gastric, pancreatic, hepatic and lung (Chrostek, 2003). Inhibition of oxidative ethanol degradation pathway induces both hepatic and pancreatic fatty acid ethyl esters (FAEE) levels, thereby showing that oxidative and

nonoxidative pathways are alternative metabolically linked pathways. Pancreatic ethanol metabolism occurs mainly in the nonoxidative pathway. The oxidative routes to acetaldehyde have also been shown in the pancreas - the cytochrome P450 2E1 & alcohol dehydrogenase pathways (Chrostek, 2003). β-alanine is produced from degradation of polyamines including spermine and spermidine, in various plant and yeast (Terano, 1978). Studies in *Zea mays* radiotracer experiments have demonstrated the ability of maize shoots to convert spermidine and spermine to β-alanine and its biosynthesis intermediate 1.3- diaminopropane (Terano, 1978). Some reports showed the ability of fruit pericarp discs of tomato (*Lycopersico nesculentum*) to catabolize radiolabeled spermidine to putrescine and β-lanine" (Rastogi, 1989).

Aldehyde dehydrogenases are well known for their involvement in biological processes in both prokaryotic and eukaryotic organism. They are up-regulated in both abiotic and biotic stress responses. It is highly associated with ROS and oxidative response in living organism that are faced with stress. They are also known for their ability to lower oxidative stress, mainly cause by aldehydes (Vasilio & Papa 2009; Chen *et al*., 2007). It has also been reported that ALDH are up-regulated in response to heat, salinity, dehydration, UV radiation and pesticides or metals (Chen *et al*., 2007). Aldehyde dehydrogenase has only been associated with abiotic stress. ALDH may function as a regulator of oxygen reactive species produced by the Hypersensitive Response during aphid infestation.

The contitutively expressed proteins in wheat-aphid infestation identified by LC-MS/MS:

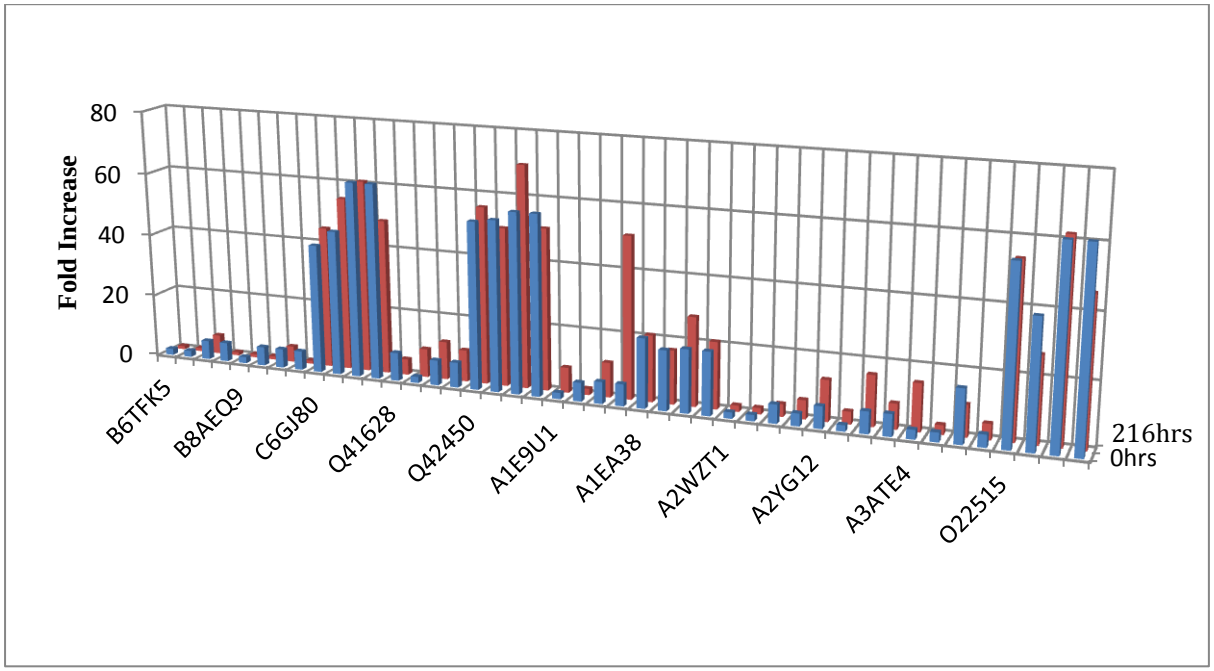

Figure 3.9: A graphical representation of the constitutively expressed proteins identified in both RWA infestated and control wheat plants. Eleven proteins were identified as being produced continuously whether the plant is subjected to stress or not. The proteins were identified as elongation factor Tu (B6TFK5), putative uncharacterized protein (B8AEQ9), photosystem II cp47 chlorophyll apoprotein (C6GJ80), ATP/ADP carrier protein (Q41628), ribulose bisphosphate carboxylase/ oxygenase activase B (Q42450), Cythochrome b559 subunit alpha (A1E9U1), chytochrome b6 (A1EA38), putative uncharacterized protein (A2WZT1, A2YG12, A3ATE4) and glycine carboxylase (O22515). Almost all the proteins that were constitutively induced were related to energy metabolism.

Pathways involved in Tugela and Tugela DN wheat response to aphid infestation or the constitutively expressed proteins

L-ascorbate peroxidase pathway involvement in wheat-aphid infestation

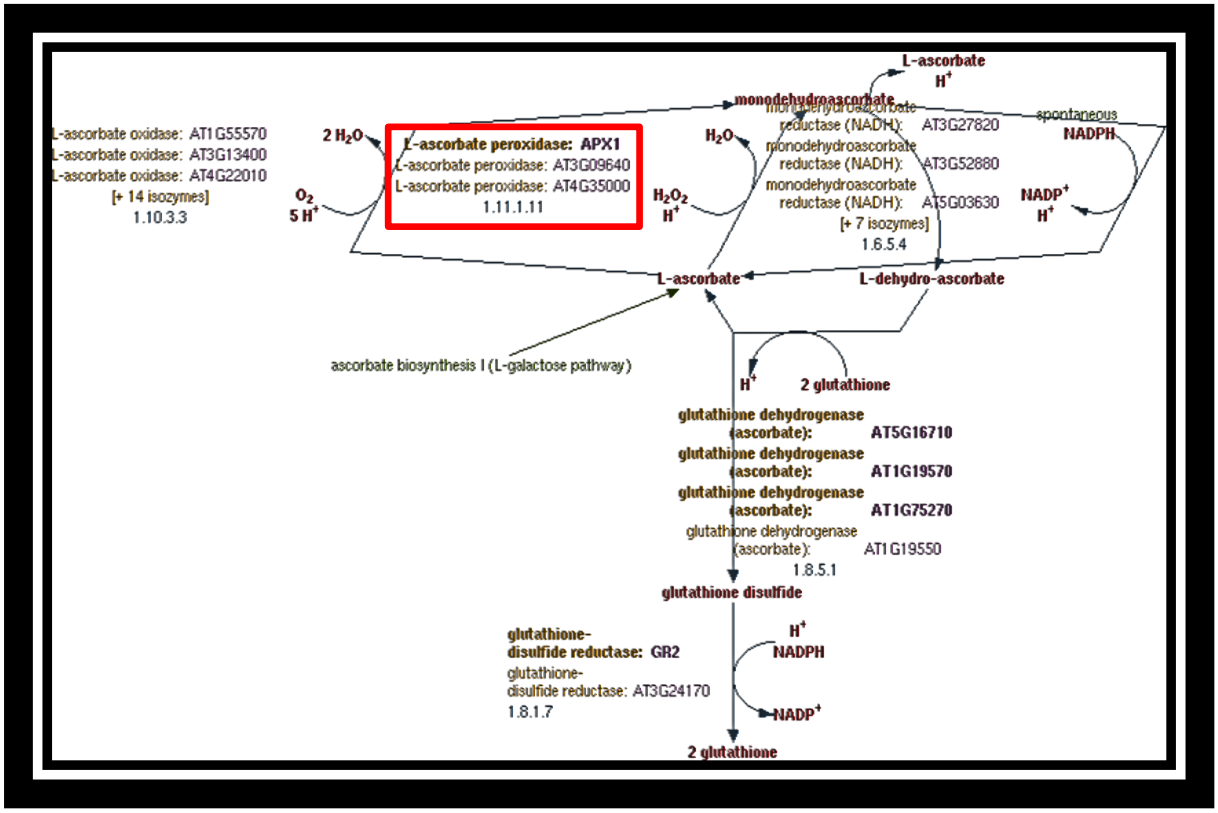

Figure 3.10: A graphical representation of ascorbate peroxidase in the ascorbate glutathione cycle (taken from MetaCyc: www.**metacyc**.org; Caspi *et al.,* 2012)

Ascorbate + H_2O_2 = dehydroascorbate + $2H_2O$:

The antioxidant function of catalases is controlled by ascorbate peroxidases (APX). The peroxidase uses ascorbic acid as a hydrogen donor, in the breaking down of hydrogen peroxide (Asada, 2006). The peroxidases are contained in cell compartments such as the stroma, thylakoid membrane (tAPX), the mitochondria (mAPX) and the cytosol (cAPX) (Asada, 1992; Ishikawa *et al*., 1998). There are two chloroplastic APX isoenzymes that are encoded by the APXII gene, in which the isozoenzymes′ mRNAs are regulated by splicing of their terminal exons (Yoshimura *et al*., 1999). Different APX respond to stress in a different manner. APX has been reported to have two cytosolic forms which are membrane bound and have pure stress defence roles. They also have a function in scavenging hydrogen peroxide

(Davletova *et al*., 2005). Several proteomic studies showed an up-regulation in different isoforms of APX and other peroxidases, under drought (Hajheidari *et al*., 2005), salinity (Yan *et al*., 2005), high temperature (Su¨le*et al*., 2004), Cd stress (Sarry *et al*., 2006b), Mn stress (Fecht-Christoffers *et al*., 2003) and ozone stress (Agarwal *et al*., 2002). The Hypersentive Response (HR) is known to be activated during pathogen infections and generates an oxidative burst by producing reactive oxygen species (ROS), superoxide anions, hydrogen peroxide, hydroxyl radicals and nitrous oxide, which trigger the deposition of lignin and callose, as well as the cross-linking of pre-formed hydroxyproline-rich glycoproteins such as P33 to the wall matrix via the tyrosine in the PPPPY motif (Pontier, D,; Balague C, Roby D., 1998). It is also known that there is cross-talk between plants responses to pathogens and wounding (as in the case of aphid probing) (Kunkel & Brooks, 2002). Therefore ascorbate peroxidase may play a role in the regulation of the level of these reactive oxygen species in the wheat response to aphid infestation.

Glycine decarboxylase pathway involvement in wheat-aphid infestation

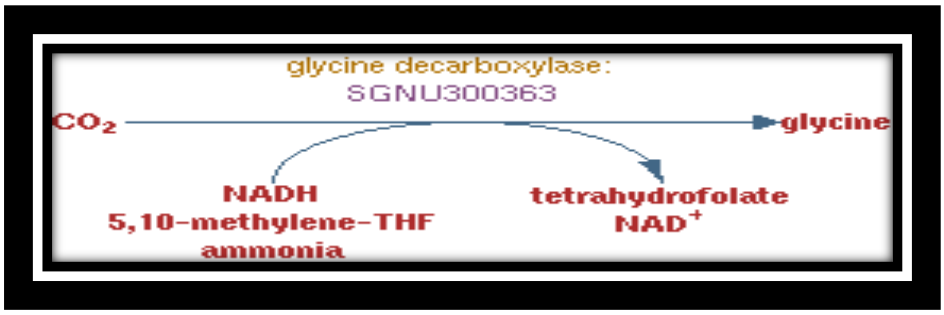

Figure 3.11: A graphical representation of the involvement of glycine decarboxylase in the *Coffea canephora* glycine biosynthesis II pathway (taken from MetaCyc: www.**metacyc**.org; Caspi *et al.,* 2012)**.**

Glycine decarboxylase, together with Serine hydroxymethyltransferase, is responsible for the conversion of Gly to Ser during photorespiration. Up-regulaton of glycine decarboxylase was shown in *Arabidopsis thaliana* stressed by s-nitrosylation and glutathionylation (Palmieri *et al*., 2010). Glycine decarboxylase is a part of photorespiratory system. Inhibition of Glycine decarboxylase by NO donor S-nitrosoglutathione has been reported to result in the increased accumulation of ROS which leads to oxidative damage of glycine decarboxylase (Taylor *et al*., 2002). It has been described that oat glycine decarboxylase is involved in the response to the toxin victorin produced by the fungus *Cochliobolus victoriae* (Navarre and Wolpert, 1995; Yao *et al*., 2002). In this study Glycine decarboxylase was induced in wounding

stresses caused by RWA. Therefore a further investigation is required in understanding the regulation of glycine decarboxylase in response to wounding stress or aphid infestation.

Phosphorylated proteins identified by nanoLC-MS/MS:

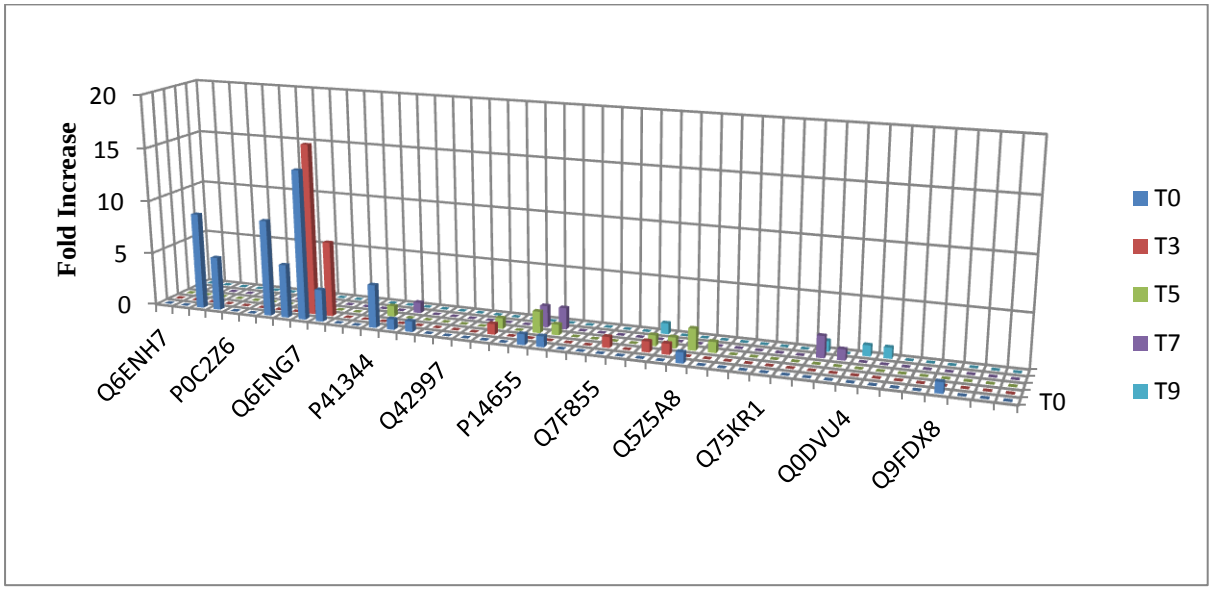

Figure 3.12: A graphical representation of the differential abundance of phosphopeptides that were expressed in response to aphid infestation of wheat plants. These proteins were up-regulated or down-regulated in either Tugela or Tugela DN plants when infested with RWA for 216 hrs. The first column represents the Tugela DN stressed protein, 2nd Tugela DN (control), 3rd Tugela (test) and 4th Tugela (control) per accession number. The proteins were identified as ATP synthase subunit alpha (Q6ENH7), ATP synthase subunit alpha (P0C2Z6), ATP synthase subunit beta (Q6ENG7), Ferredoxin NADP reductase leaf isoenzyme (P41344), Ferredoxin-nitrite reductase/chloroplastic (Q42997), hypothetical protein (P14655), Peroxiredoxin -2E -2 (Q7F855), photosystem II stability/ assembly factor HCF136 (Q5Z5A8), pyruvate / phosphatase dikinase (Q75KR1), zinc finger cccH domain-containg protein 20 (Q0DVU4), and zinc finger protein (Q9FDX8).

The phosphosproteins identified in Figure 3.11 are likely to be involved in the resistance mechanism of Tugela DN because of their up or down regulation in aphid infested and control leaves in either Tugela DN or Tugela plants. Some of the peptides were present in only Tugela DN extracts (infested and control plants) at the beginning of the RWA infestation, and were down regulated as the infestation persisted, while not being expressed in the Tugela extracts. Other peptides were not expressed in either the Tugela DN or Tugela extracts, at the start of the infestation but were up-regulated in Tugela DN over time of infestation while remaining unexpressed in the Tugela extracts. Similar results were found for proteins expressed in Tugela only and not in Tugela DN extracts. Since these proteinS

are expressed only in Tugela DN or only in Tugela, they must be related to the resistance mechanism of Tugela DN.

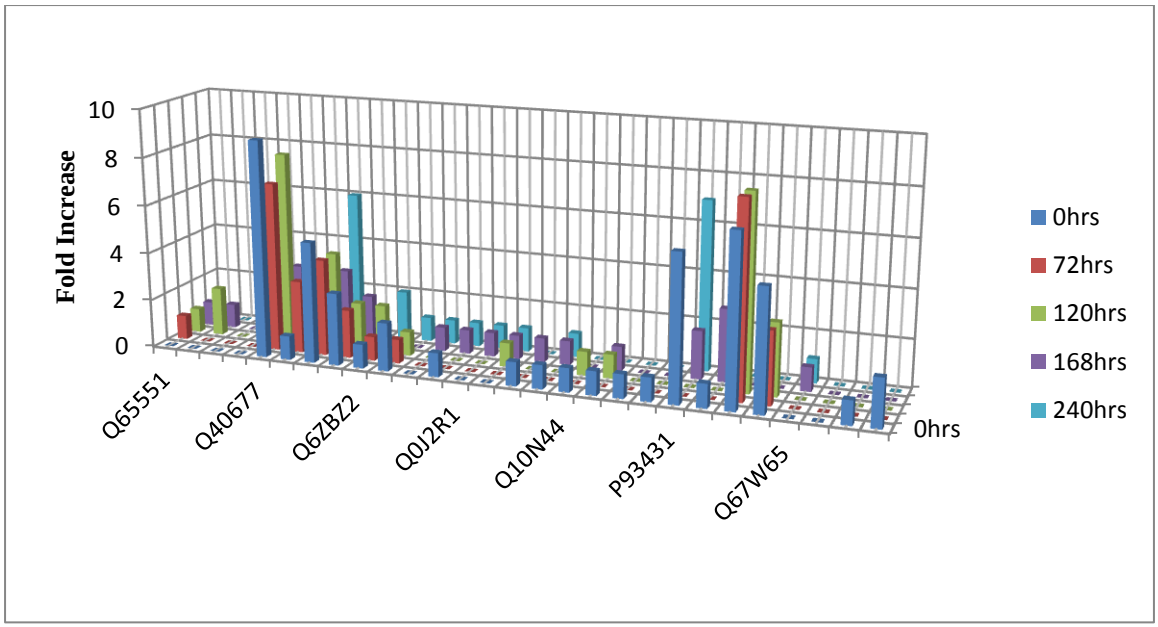

Figure 3.13: A graphical representation of the differential abundance of phosphopeptides expressed in response to aphid infestation of wheat plants. These are the phosphopeptides that where continuously expressed over time of infestation or were expressed in both the Tugela DN and Tugela extracts whether infested with RWA or not. Seven peptides were not regulated by stress as they were always present whether there was infestation or not. These peptides were: ATP-dependent zinc metallo-protease FTSH2 (Q65551), fructose-bisphosphate aldolase (Q406677), Germin like protein (Q6ZBZ2), probable protein phosphatase (Q0J2R1), protein IN-21 homolog A (Q10N44), Ribulose bisphosphate carboxylase/oxygenase activase (P93431) and lastly Transcription initiation factor (Q67W65).

The following phosphoprotein pathways are therefore involved in the wheat response to aphid infestation or wheat's resistance mechanism:

Ferredoxin-NADP$^+$ reductase leaf isoenzyme pathway was found to be related to

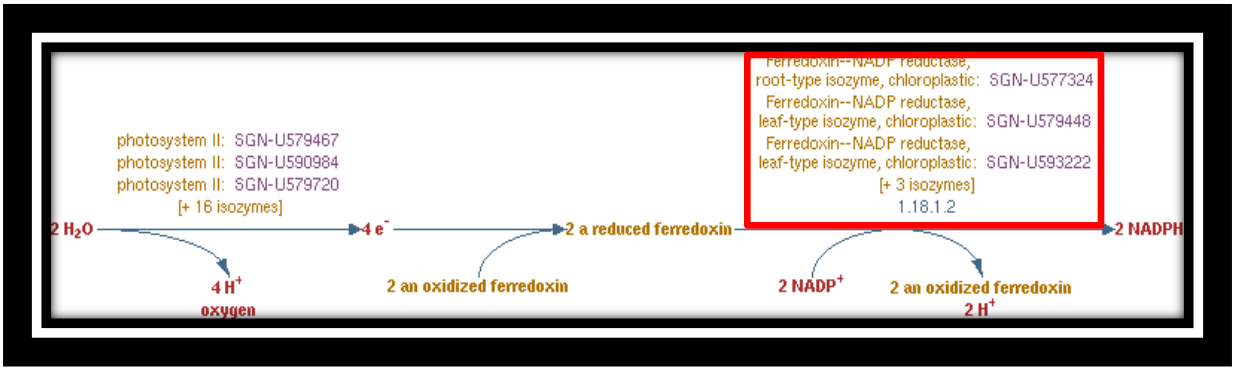

Figure 3.14: The involvement of ferredoxin-NADP$^+$ reductase leaf isoenzyme in photosynthesis light reactions: TOMATO (taken from MetaCyc: www.**metacyc**.org; Caspi *et al.,* 2012**).**

Ferredoxin NADP$^+$ Reductase is involved in electron transfer chains that are involved in metabolic processes such as photosynthesis, nitrogen fixation, isoprenoid biosynthesis, steroid metabolism, xenobiotic detoxification, oxidative-stress response, and iron–sulphur cluster biogenesis. Photosynthesis has two processes, the light reactions and the dark reactions. The light reactions occurs in the photosystem I and photosystem II, where light energy is harvested and is used to power the transfer of electrons from water, via a series of electron donors and acceptors, to the final acceptor NADP$^+$, which is reduced to NADPH. The NADPH that is generated from light reactions is used in the dark reaction for the sugar synthesis. However proton motive force across the thylakoid membrane and the proton gradient used in the ATP synthesis are generated in the light reactions. For instance, knocked-down plants in which the levels of either FNR or Fd had been decreased by RNA antisense or RNA interference approaches display enhanced susceptibility to several sources of stress, in addition to growth and reproductive penalties (Hajirezaei *et al*., 2002; Blanco *et al*., 2011).

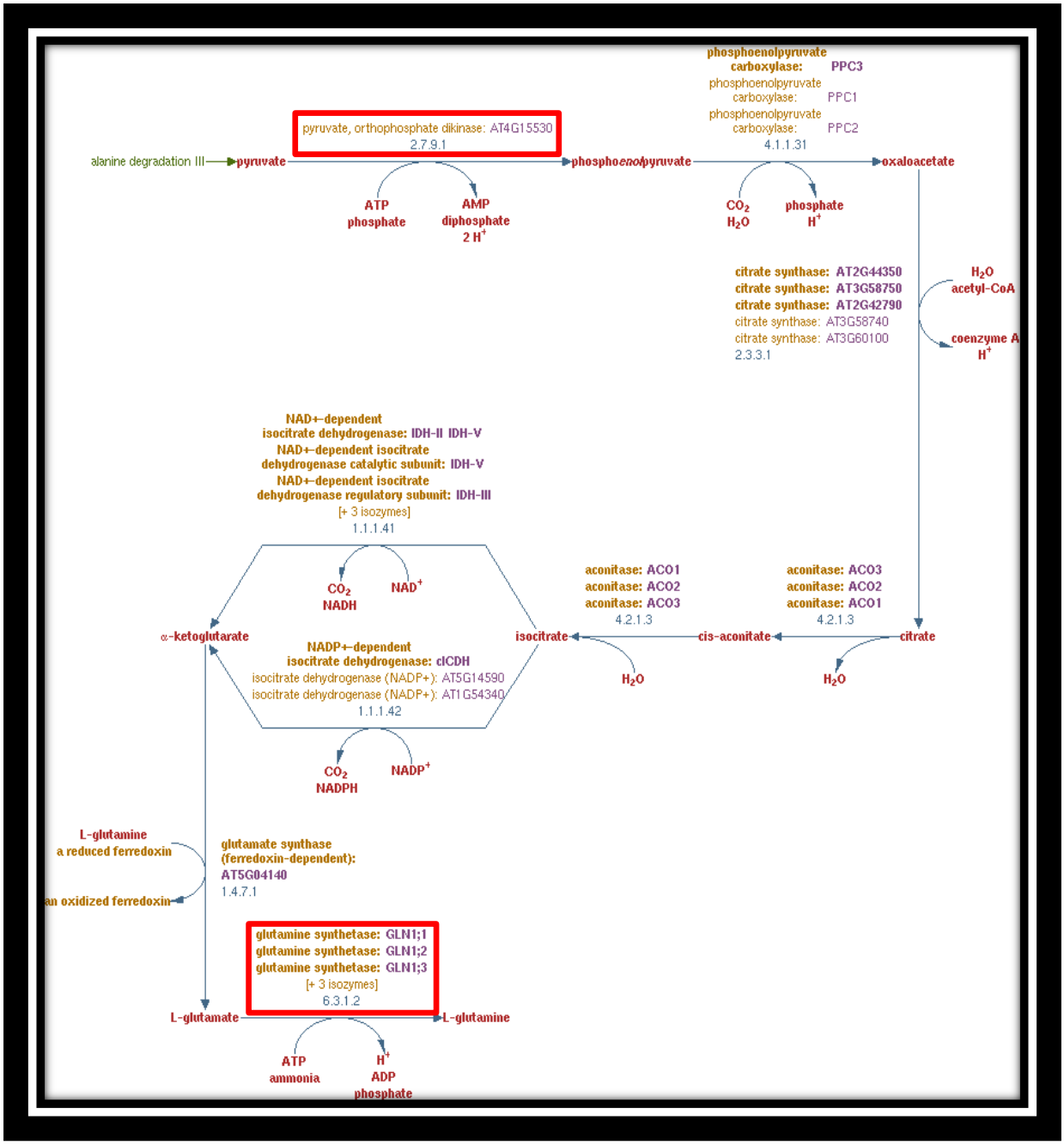

Figure 3.15: Arabidopsis thaliana col Pathway: glutamine biosynthesis III (taken from MetaCyc: www.**metacyc**.org; Caspi *et al.*, 2012)**.**

A large fraction of this protein content may be derived from the remobilization of nitrogen from proteins in senescing leaves (Hirel, 2001; Barneix, 2007; Donnison, 2007). This nitrogen can be transported in the form of amino acids, principally glutamine, through the phloem. Improving the efficiency of this process could lead to the production of seed-based crops with higher protein levels (Taylor, 2010). One of the enzymes in this pathway, pyruvate, orthodiphosphate dikinase (PPDK), plays a prominent role in photosynthesis in C4

plants such as *Zea mays*(maize), yet it is also expressed in C3 plants, such as *Arabidopsis thaliana* col . This pathway may help to explain the physiological function of PPDK in C3 plants (Taylor, 2010). Interestingly, in all plant species studied to date, both a cytosolic and a chloroplastic isoform of the PPDK enzyme are generated from alternative promoters upstream of the same chromosomal locus. Over-expression of the cytosolic form of this enzyme in Arabidopsis during senescence can lead to an increase in seed weight and seed nitrogen content indicating that this form of the enzyme may be important for nitrogen remobilization (Taylor, 2010). Pyruvate, orthophosphate dikinase is the enzyme that is involved in the photosynthetic pathway, where it is responsible for catalyzing the ATP- and independent formation of phospho*enol*pyruvate (PEP), the primary CO_2 acceptor molecule, from pyruvate. It is a rate limiting enzyme in the C_4 plants cycle and its activity is regulated in a reversible light-dependent manner, thus the pathway functions in net CO_2 assimilation (Hatch, 1987; Furbank *et al*., 1997). Pyruvate, orthophosphate dikinase regulatory protein catalyses the phosphorylation / dephosphorylation cycle in C_4 plants (Burnell and Hatch, 1984, 1985, 1986; Roeske and Chollet, 1987; Ashton *et al*., 1990). Pyruvate, orthophosphate dikinase and its regulatory protein are both located in the chloroplast stroma of the C_4 plants. The regulatory protein has been referred to as bi-functional in a sense that it catalyses both Pyruvate, orthophosphate dikinase phosphorylation and dephosphorylation. This function of Pyruvate, orthophosphate dikinase regulatory protein makes it unique because normal regulation of phosphorylation involves either kinases or phosphatases but rarely both. It also uses ADP instead of ATP as the phosphoryl donor and employs a Pi-dependent, inorganic pyrophosphate (PPi)-forming phosphorolytic dephosphorylation mechanism, as opposed to simple hydrolysis as in most protein phosphatases.

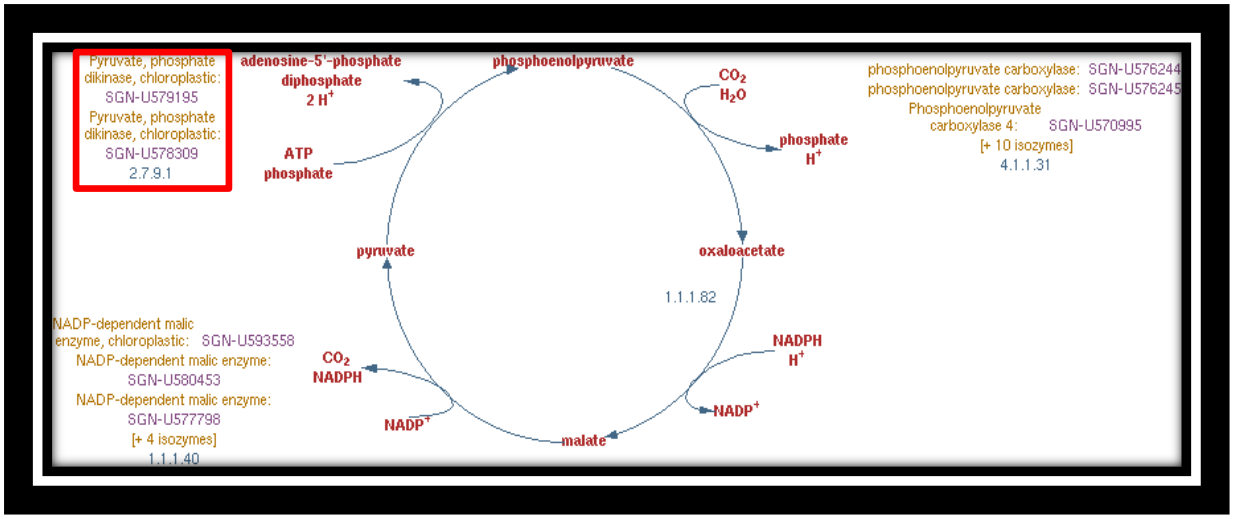

Figure 3.16: Pyruvate phosphate dikinase involvement in Solanum lycopersicum C4 photosynthetic carbon assimilation cycle pathway (taken from MetaCyc: www.**metacyc**.org; Caspi *et al.,* 2012)**.**

RUBISCO (ribulose bisphosphate carboxylase/oxygenase) has both carboxylase (photosynthetic CO_2 fixation) and oxygenase (photorespiration) activity. The carboxylase activity requires high CO_2/O_2 ratio to fix CO_2, whereas the oxygenase activity acts at a decreased CO_2/O_2 ratio. At the time of RUBISCO′s first synthesis/development, atmospheric CO_2 concentration was very high and favoured carboxylase activity. However, later in evolution, atmospheric O_2 began to rise and RUBISCO oxygenase activity increased while carboxylase activity decreased. At current CO_2 levels, photorespiration can reduce photosynthesis by more than 40%. C4 plants have evolved to response to lowered CO_2 level. The C4 photosynthetic carbon assimilation cycle transports CO_2 from mesophyll cells to bundle sheath cells via C4 acids and generates a much higher concentration of CO_2 in the bundle sheath cells. The result is a suppression of photorespiration and the maximization of photosynthesis. There are three variations of the C4 photosynthetic carbon assimilation cycle of which one is the so-called NADP-ME (NADP-malic enzyme) variant, found in major C4 crops such as maize, sorghum, and sugar cane.

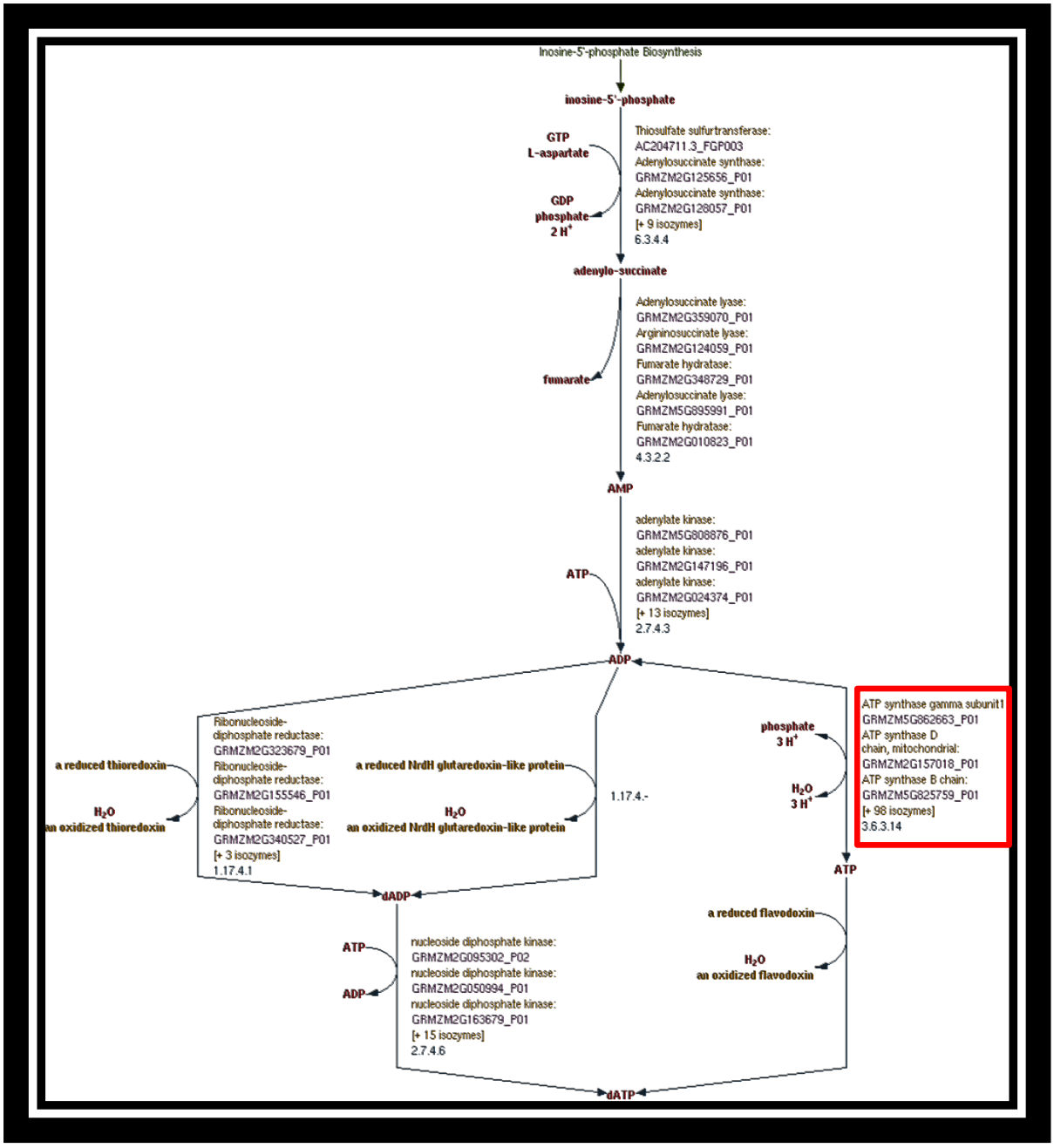

Figure 3.17: A graphical representation of ATP synthase from Zea Mays (taken from MetaCyc: www.**metacyc**.org; Caspi *et al.*, 2012)**.**

ATP synthase is devoid of redox-active prosthetic groups and is not involved in any redox functions. The regulation of the chloroplast ATP synthase by the redox state is well known (McCarthy *et al*., 2005) and for the mitochondrial enzyme indications for a redox regulation have also been shown (Wang, 2011).

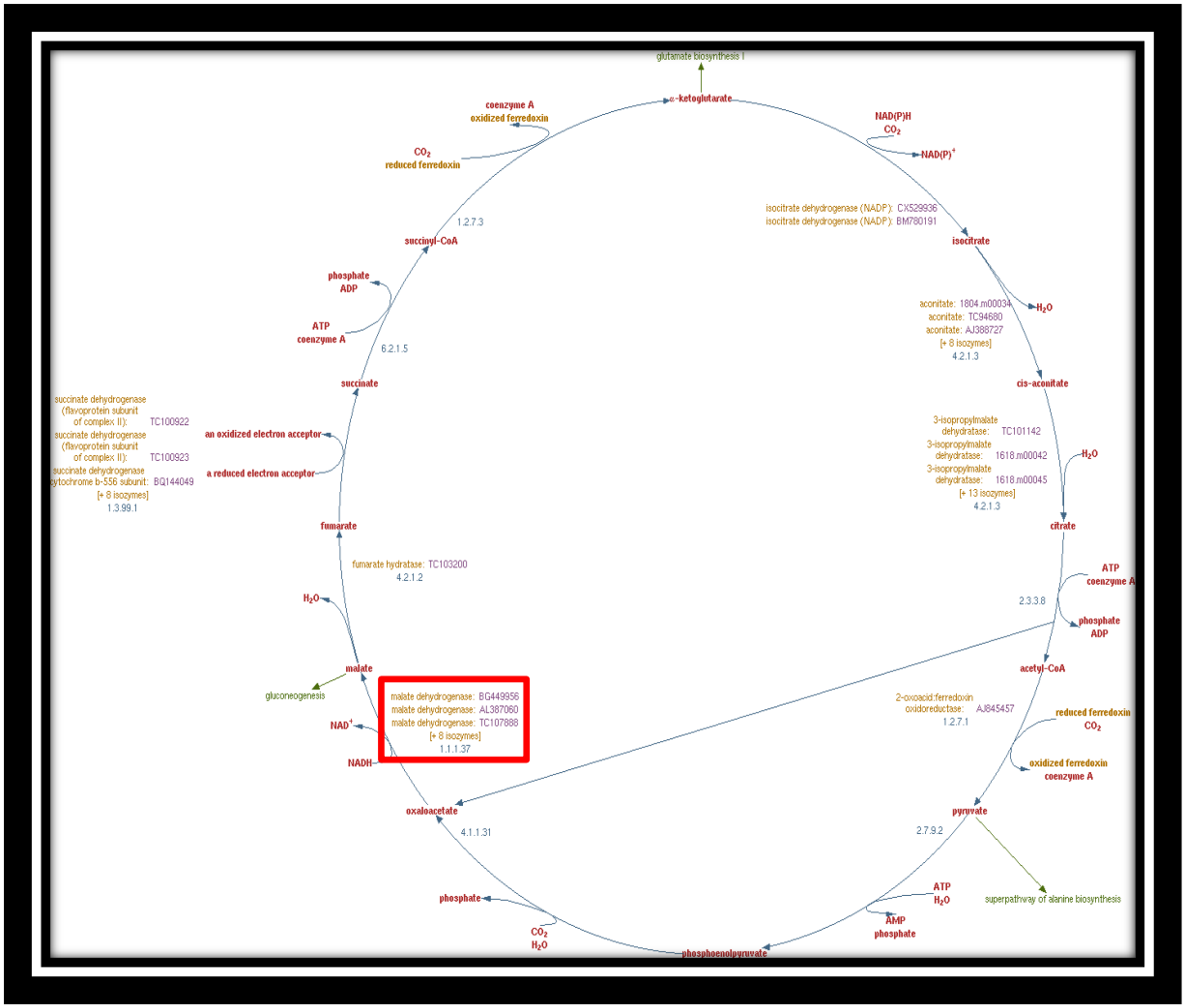

Figure 3.18: Involvement of malate dehydrogenase in reductive tricarboxylic acid cycle pathway in *Medicago truncatula* (taken from MetaCyc: www.**metacyc**.org; Caspi *et al.,* 2012)**.**

The reductive tricarboxylic acid (TCA) cycle is a carbon dioxide fixation pathway found in autotrophic eubacteria and archaea (there is a report of the pathway also operating in a strain of the green algae *Chlamydomonas reinhardtii* (Chen, 1992)). "Malate dehydrogenase (MDH) (EC 1.1.1.37) catalyzes the conversion of oxaloacetate and malate. This reaction is important in cellular metabolism, and it is coupled with easily detectable cofactor oxidation/reduction" (Musrati *et al*., 1998). Malated dehydrogenase has been shown to be up-regulated in response to various abiotic stresses in plants (Ivanishchev, 1997; Kingstonsmith *et al*., 1997). NAD-malate dehydrogenase regulation has been associated with drought stress in C_3 plants (Ivanishchev, 1997). The chloroplastic malate dehydrogenase has a vital role in C_3 plants, it shuttles reducing equivalents between the chloroplast and the cytosol of the plant

(Schiebe, 1990). Ritambhara *et al*. (2000) reported the sensitivity of malate dehydrogenase to sodium chloride (NaCl) or salt stress compared to mitochondrial malate dehydrogenase. NADP-malate dehydrogenase is also up-regulated in response to light stress, and it been suggested that it is structurally more vulnerable to attack by oxidizing agents or free radical species compared to other malate dehydrogenases (Schiebe, 1990)

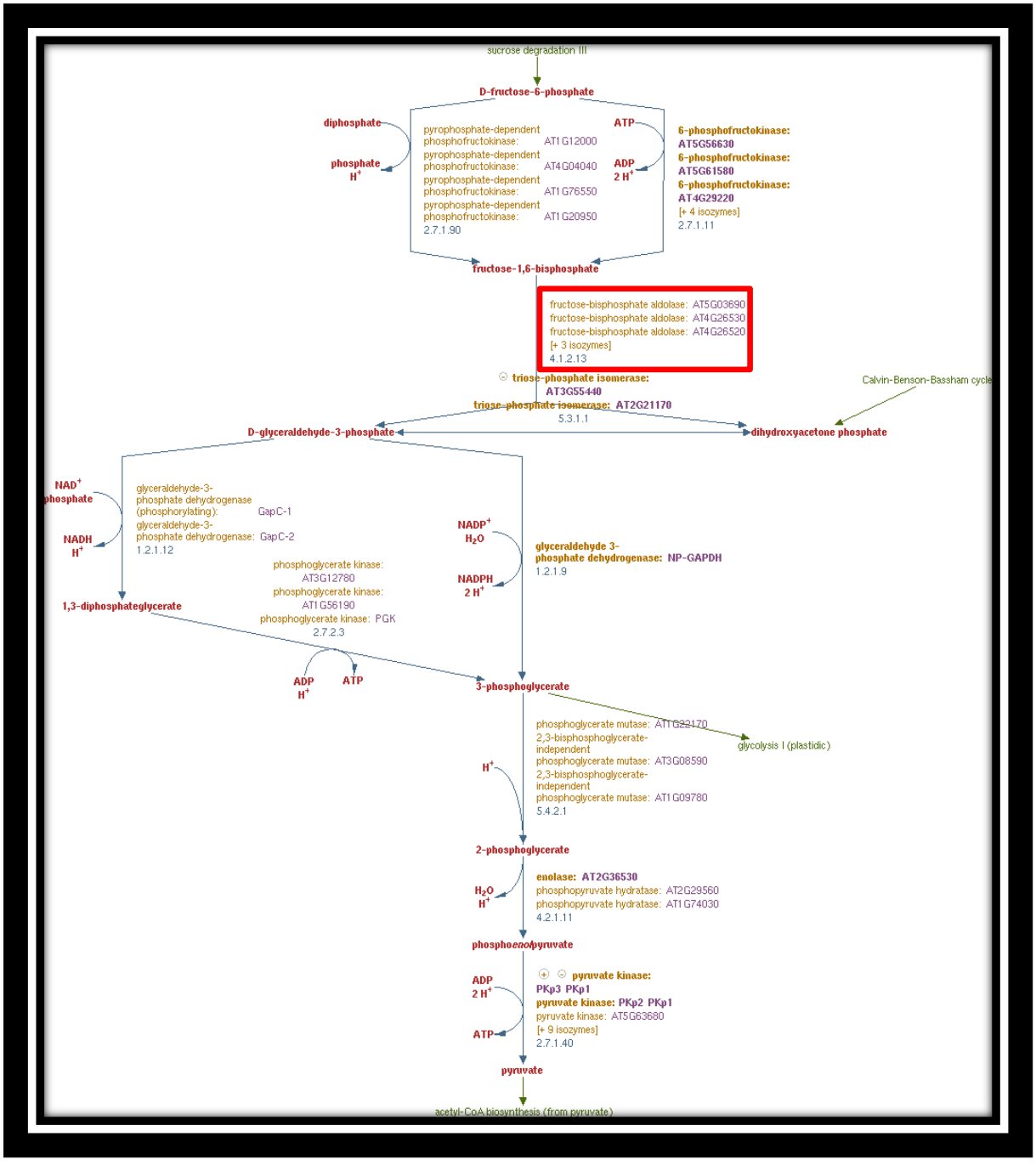

Figure 3.19: Involvement of fructose-bisphosphate aldolase in arabidopsis thaliana col pathway: glycolysis iv (plant cytosol) (taken from MetaCyc: www.**metacyc**.org; Caspi *et al.,* 2012**).**

Glycolysis is known as the central metabolic pathway that occurs in one or all parts of all organisms. it evolves anaerobic pathway that plays an important role the oxidation of hexoses to generate ATP , reductants and pyruvate ; secondly it is an amphibolic pathway (pathway that involves both catabolism and anabolism) because it can reversibly produce hexoses from various low-molecular weight molecules. Unlike in animals, in plants glycolysis is the major pathway increasing plant′s respiration because, their mitochondria hardly require fatty acids. The glycoysic pathway occurs in the cytosol and plastids, which are the sites of sucrose degradation III and starch degradation. Whereas the *plastidic* glycolysis pathway is identical to the conventional microbial glycolysis, the *cytosolic* pathway (Fig. 3.20) is slightly modified. These pathways can interact with each other through the action of highly selective transporters that are present in the inner plastid envelope (Emes, 1993). Glycolysis I that occurs in the plastids of nonphotosynthetic tissues has the primary function in starch degradation to generate carbon skeletons, reductants and ATP for anabolic pathways such as that of fatty acid biosynthesis initiation I via acetyl-CoA biosynthesis (from pyruvate) The same products are also generated in the cytosol from the degradation of sucrose via glycolysis IV (plant cytosol). In developing oil seeds, phosphor-enoyl-pyruvate derived from glycolysis IV is transported from the cytosol to plastids, where it is further converted to pyruvate then acetyl-CoA to fuel fatty acid and oil biosynthesis.

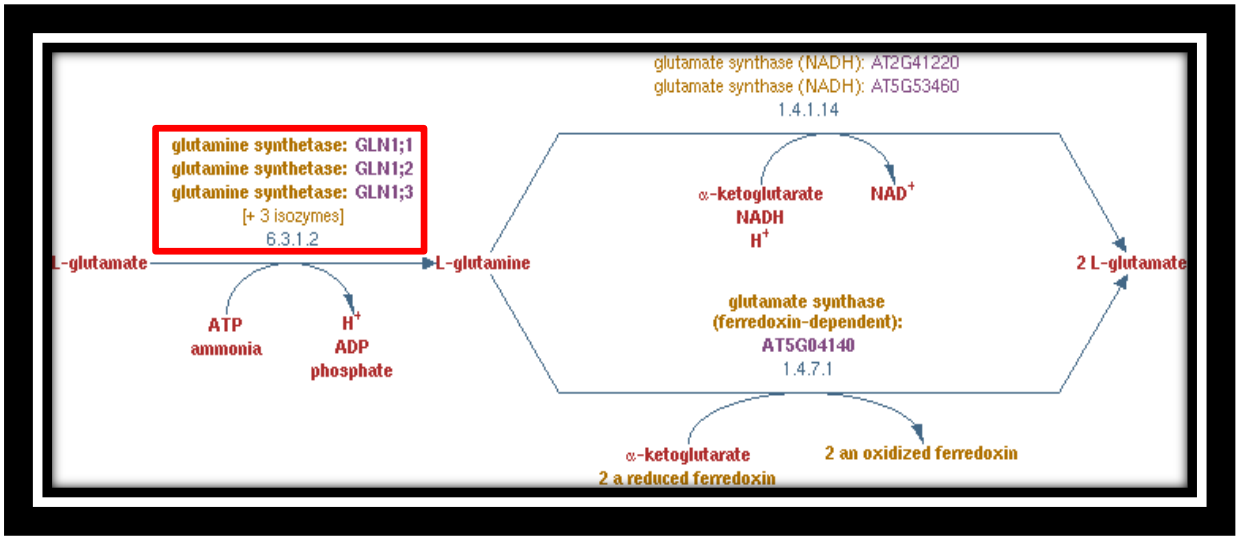

Figure 3.20: Involvement of glutamate synthase in *Arabidopsis thaliana* col ammonia assimilation cycle II (taken from MetaCyc: www.**metacyc**.org; Caspi *et al.,* 2012**).**

In plants the primary ammonia assimilation is via nitrogen fixation and nitrate assimilation. In addition to these primary routes, ammonia is also liberated from photorespiration, protein

and amino acid metabolism, and the breakdown of nitrogen transport compounds. The rate of ammonia generation during photorespiration is about 10 times the rate of nitrate assimilation. The vast amount of ammonia released from photorespiration is immediately re-assimilated via the ammonia assimilation cycle where ammonia is stored in the form of glutamate at the expense of ATP. This is achieved by the two concerted reactions catalyzed by glutamine synthetase and glutamate synthase (Miflin, 2002). Abiotic stresses such as salinity, results in intracellular hyperammonia and toxicity if not removed efficiently (Lutts *et al*., 1999). Glutamate synthase incorporates ammonium ions into glutamine and glutamate. Studies have reported light response as responsible for the upregulation of ferredoxin dependent glutamate synthase activity in cytledons and leaves in a range of plants as a result of mRNA synthesis and protein synthesis (Ireland & Lea, 1999). In arabidopsis, upregulation of ferredoxin-glutamate synthase GLU1 mRNA was observed in response to light stress (Coschigano, 1998). The activity of ferredoxin dependent glutamate synthase is affected by the availability of nitrogen sources which may also be related to light availability (Ireland & Lea, 1999; Suzuki &Rothstein, 1997). High induction of glutamate synthase mRNA was observed in barley grown in the light on nitrate (Pajuelo *et al*., 1997). Up-regulation of glutamate synthase was also indicated in soybean roots supplied with ammonium nitrate in the dark. During the development and expansion of a new leaf, Fd-glutamate synthase activity has been shown to increase with the onset of photosynthesis and photorespiration (Emes &Tobin, 1993).

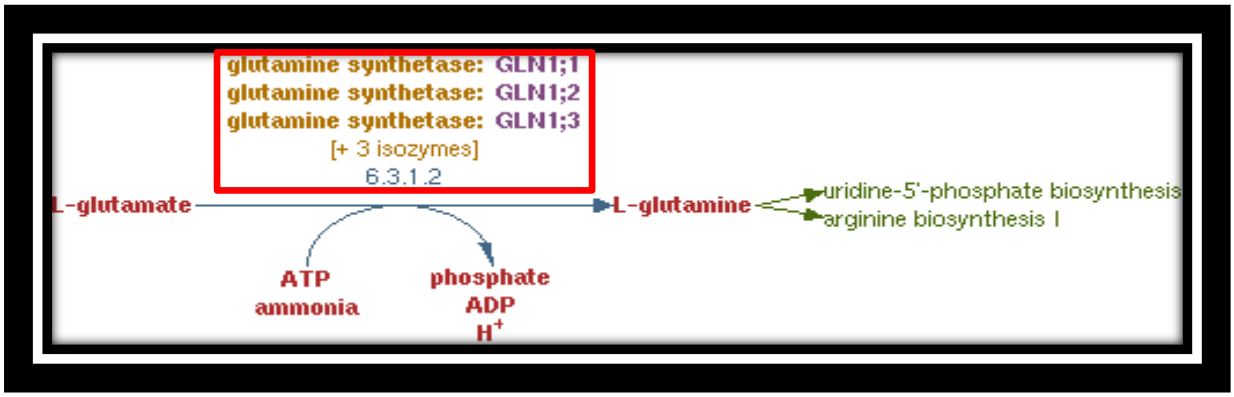

Figure 3.21: Representation of glutamine synthetase involvement in *Arabidopsis thaliana* col glutamine biosynthesis I pathway (taken from MetaCyc: www.**metacyc**.org; Caspi *et al.,* 2012)**.**

In addition to being a building block for protein synthesis, glutamine, along with glutamate, asparagine, and aspartate are used to transport nitrogen throughout the plant, and they

represent 70% of the total free amino acids in Arabidopsis. Nitrogen is first assimilated from the soil into glutamine and glutamate via glutamine synthetase and glutamate synthase, and later into aspartate and asparagine via aspartate aminotransferase and asparagine synthetase. Plants have two types of glutamine synthetase isoenzymes: one located in the cytosol (GS1) and the other in the chloroplast (GS2). The Arabidopsis GS1 is encoded by a small family of five genes, whereas GS2 is encoded by a single gene. GS1 is the major form of glutamine synthetase detected in the root. In leaves, both GS1 and GS2 are abundant. GS1 is induced by sucrose. GS2 is induced by light (indeed phytochrome) and sucrose. The sucrose-inductions of GS1 and GS2 are at both mRNA and enzyme activity levels. In contrast, amino acids (aspartate, asparagine, glutamate and glutamine) were shown to have an antagonistic effect on the sucrose-induction of GS1 and GS2. Thus, the metabolic regulation of GS1 and GS2 in plant is controlled by the relative ratio of carbon versus nitrogen (Oliveira, 1999).

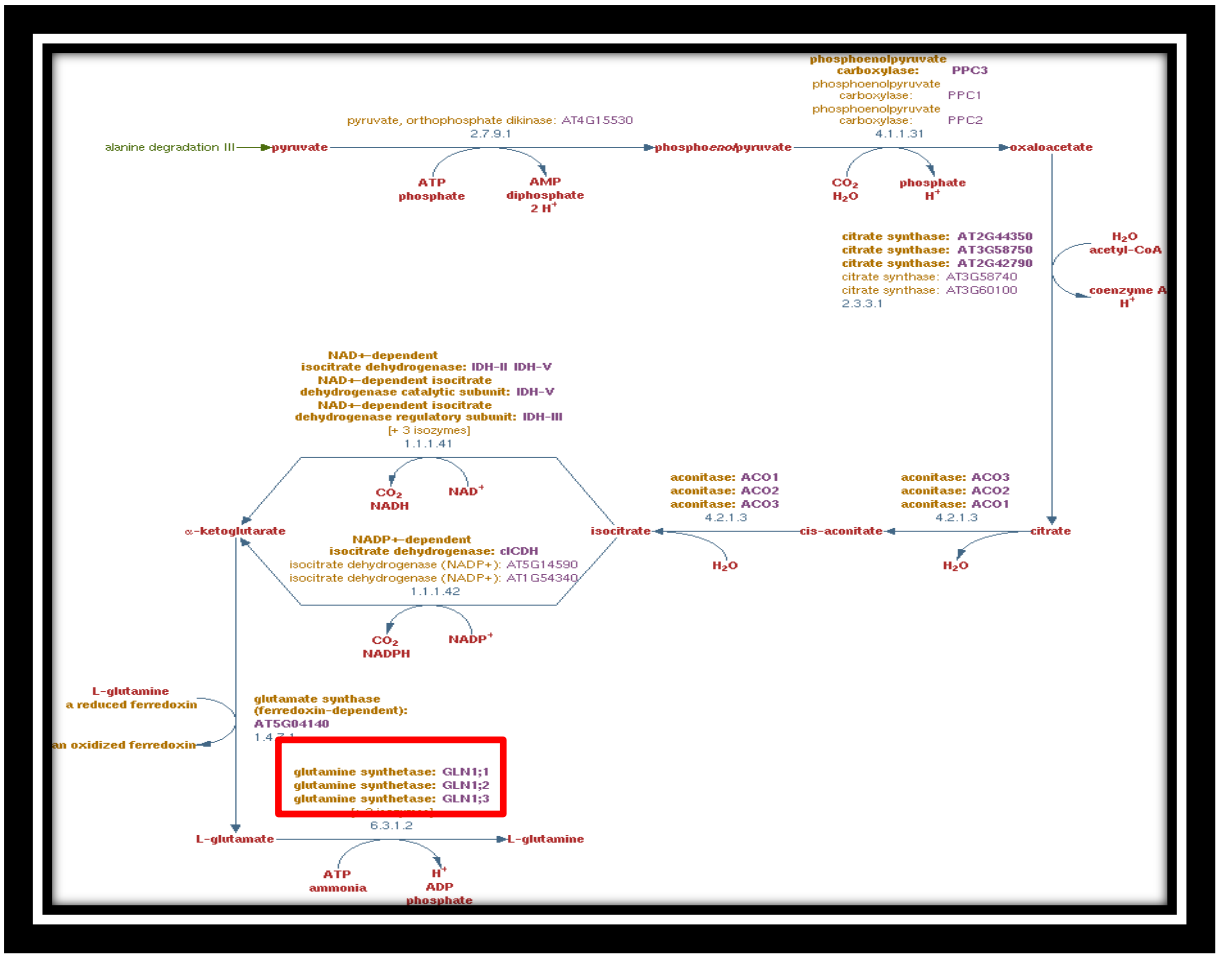

Figure 3.22: A graphical representation showing the involvement of glutamine synthetase in *Arabidopsis thaliana* col Pathway (taken from MetaCyc: www.**metacyc**.org; Caspi *et al.,* 2012)**.**

Ammonium that is derived from primary nitrate reduction, as well as other metabolic pathways such as root uptake, photorespiration and amino acid catabolism, is converted into glutamine by glutamine synthetase and then to glutamate by glutamate synthase (Teixeira and Fidalgo, 2009). Glutamine synthetase has a key role in ammonium induction in plants. It catabolizes the ATP-dependent conversion of Glutamate into Glutamine while scavenging ammonium. There are two types of glutamate synthetases present in higher plants and are categorised based on their cellular localizations. Cytosolic glutamine synthetase (GS1) is a cytosolic form of glutamine synthetase in plant roots while plastidic glutamine synthetase (GS2) is a soluble protein in the chloroplast and the mitochondria for ammonium scavenging during photorespiration (Taira *et al*., 2004). Several glutamate synthetase 1 isoenzymes were identified in higher plants. GS1 numbers differ in different plant species, thus maize has five isoenzymes of GS1(*GLN1-1*, *GLN1-2*, *GLN1-3*, *GLN1-4* and *GLN1-5*) (Martin *et al*., 2006); rice plants possess three *GS1* genes (*OsGS1;1*, *OsGS1;2* and *OsGS1;3*) (Tabuchi *et al*., 2005); and in Arabidopsis, five putative genes for GS1 have been identified, (*GLN1;1*, *GLN1;2*, *GLN1;3*, *GLN1;4* and *GLN1;5*) (Ishiyama *et al*., 2004a). Up-regulation of various glutamate synthases1 in response to various abiotic stress was reported, these include cold (Kwon *et al*., 2007), salt (Debouba *et al*., 2006), and H_2O_2 (Scarpeci *et al*., 2008). In the case of cytosolic GS, accumulating studies report an induction of GLN1 both at the RNA and protein levels under various stress conditions (Debouba *et al*., 2006; Kwon *et al*., 2007). Finnemann and Schjoerring (2000) reported post-translationally regulation of GS1in *Brassica napus* by reversible phosphorylation and that both protein kinase and protein phosphatase are able to catalyse the reactions (Finnemann and Schjoerring, 2000). The same type of post-translational modification was also observed for Arabidopsis; cytosolic glutamine synthetase by calcium-dependent protein kinase -related protein kinase (Li *et al*., 2006).

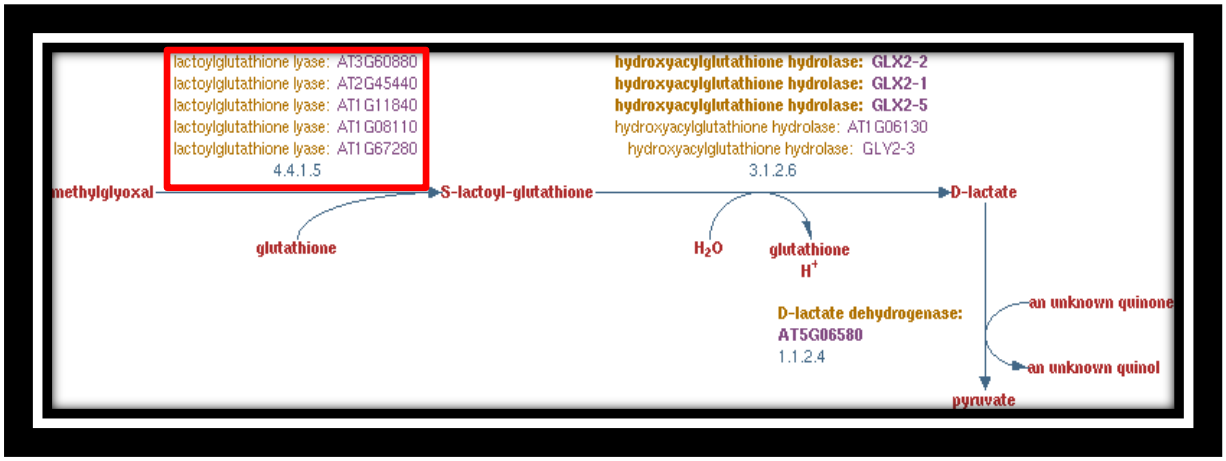

Figure 3.23: A graphical representation of lactoylglutathione lyase involvement in *Arabidopsis thaliana* col methylglyoxal degradation1 pathway (taken from MetaCyc: www.**metacyc**.org; Caspi *et al.,* 2012)**.**

The glyoxalase system has two enzymes, namely lactoylglutathione lyase (glyoxalase I and hydroxyacylglutathione hydrolase (glyoxalase II)). These enzymes act coordinately in the conversion of a variety of α-keto aldehydes into hydroxyacids in the presence of glutathione. Glyoxalase I, a Zn(II) or Ni(II) metalloprotein, has the ability of forming (2-hydroxyacyl)-glutathione from a variety of α-ketoaldehydes in a reaction that involves glutathione. Glutathione thiolesters are therefore hydrolyzed by glyoxalase II to form free hydroxyacids and simultaneously regenerate glutathione. The glyoxalase system has been found in all organisms studied to date; but its exact role in the cell has still not been clearly defined. Glyoxalase I and II can utilize a number of 2-oxoaldehydes, the primary physiological substrate of the glyoxalase system which appears to be methylglyoxal (MG), a cytotoxic compound formed primarily as a byproduct of carbohydrate and lipid metabolism (Richard 1991, Ball 1981, Concha 1996). MG is both mutagenic and cytotoxic: it reacts with DNA to form modified guanylate residues and interstrand crosslinks; MG can also reacts with proteins to form glycosylamine derivatives of arginine and lysine and hemithioacetals with cysteines. It has been postulated that one of the main roles of the glyoxalase system is the glutathione-based detoxification of MG (Marasinghe 2005, Maiti 1997)

Lactoylglutathione lyase is up-regulated in abiotic stress responses such as salinity. It is involved in the glutathione-based detoxification of methylglyoxal (MG), a toxic endproduct of amino acid and carbohydrate metabolism. MG is produced by phosphate fragmentation and elimination from phosphor-eno-diolate and from the aminoacetone oxidation during

threonine biosynthesis (Thornalley, 1996). MG is up-regulated in abiotic stress conditions such as salinity, drought and cold (Yadav *et al*., 2005a). Overexpression of glyoxalase I, in tobacco plants exposed to salinity was reported (Silinga *et al*., 2006). The detoxification of ROS and the maintenance of the protein redox balance are crucial mechanisms in salt stress tolerance. Studies have reported the induction of two isoforms of glyoxalase I in response to aluminium stress induced tomato plants. Up-regulation of lactoylglutathione lyase transcript was also found in Al-treated tomato roots (Zhou *et al*., 2008).

3.4 Conclusions

Some studies have reported that the early plant responses to aphid or pathogen infections, share common events such as phosphorylation, membrane depolarization , calcium influx and release of reactive oxygen species (ROS) (Garcia-Burgger *et al*., 2006), thereby leading to the activation of phytohormone-dependent pathways. In pathogen infections, these are followed by ethylene and jasmonate dependent responses, activated by neucrophic pathogens (Thomma *et al*., 2009) and insects (Maffie *et al*, 2007), whereas salicylate depended response is activated by biotrophic pathogens (Thomma *et al*., 2009). Most of the pathways identified in this study, as involved in the wheat stress response to aphid infection, where linked to oxygen reactive species, indicating that it is very likely that one of the early responses of wheat to aphid infection in this induction of the hypersensitive response, as observed in pathogen infections. Which downstream pathway is subsequently activated could not be identified in this study and will have to form the focus of future studies.

Other pathways identified in the stress response where more related to sucrose and energy metabolism. Previous studies (Ibraheem *et al*., 2008; 2011; 2013) have identified a key role for Sucrose Transporter in the stress response to aphid infection, mainly as a result of loss of assimilates due to phloem damage. It is therefore not surprising that sucrose and energy metabolic pathways are involved in the stress response.

Chlorosis was observed after 48 hour infestation with aphids on Tugela (the susceptible stain), while on Tugela DN there were no visible symptoms of chlorosis until after 120 hrs of infestation. This is not a surprising observation since many of the pathways identified as possibly involved in the resistance mechanism of Tugela DN plants, were involved in maintaining the integrity of the chloroplasts and the photosynthetic systems. It would therefore be interesting in future studies to explore how the proteome of the chloroplasts

varying in susceptible versus resistant strains of wheat, to aphid infection. Future studies will also need to look at the differences in the wheat response to the newly discovered, more virulent strains of RWA i.e RWASA2 and 3. These studies could be able to then answer the question of whether pathways involved in preserving the chloroplast′s integrity and the photosynthetic systems are indeed related to the wheat′s resistance mechanism to aphid infection and how these may be overcome by newly evolving aphid species.

It is a concern that proteins such as chitanase and β-1,3 gulcanases, which are known to be involved in the plant stress response to aphids, were not identified, but this could be purely because of the time frames selected in this study for harvesting the leaves. Future studies will therefore have to look at time intervals within the first day of infection. The time intervals chosen in this study were based on previous proteomic studies (Cassandra Louw MSc thesis, Rhodes University, 2007) and observed physical and physiological changes in infected plants.

3.5 References

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CHAPTER 4:
TWO DIMENSIONAL POLYACRYLAMIDE GEL ELECTROPHORESIS AND MS/MS ANALYSES OF PHOSPHORUS-ENRICHED WHEAT PROTEINS AFTER APHID INFESTATION

4.1 INTRODUCTION

Two dimensional gel electrophoresis in combination with mass spectrometry is regarded as a powerful tool for protein separation, visualization and identification of expressed proteins in biological systems. Plant proteomics of specific plant tissues including seeds (Gallardo *et al*., 2001), mitochondria (Kruft *et al*., 2001; Millar *et al*., 2001), maize root tips (Chang *et al*., 2000), barrel medic roots (Mathesius *et al*., 2001), vacuoles (Carter *et al*., 2004), chloroplasts (Kleffmann *et al*., 2004) and thylakoids (Zolla *et al*., 2002, 2003) have been reported. Meanwhile, other researchers mainly focused on subcellular proteomes including chloroplast membranes (Peltier *et al*., 2002; Zolla *et al*., 2004), cell wall ((Borderies *et al*., 2003), nuclear envelop (Bae *et al*., 2003) and secreted proteins (Greenbaum *et al*., 2001; Hathout, 2007).

About 2,528 unique proteins were identified in rice seeds, leaf and roots in systematic proteomics analysis using 2DE followed by LC-MS/MS and MudPIT (Koller *et al*., 2002). In *Arabidopsis thaliana* a total of 27 proteins were identified using nanoLC-MS/MS in which 21 proteins were up-regulated in response to salinity treatment and 6 showed some down regulation. A defence response is elicited by SA and the processes affected concerned re-induction of the late maturation program, quality of protein translation, priming of seed metabolism, synthesis of antioxidant enzymes, and mobilization of seed storage proteins (Rajou *et al*., 2005). Combined proteomics approaches revealed the significance of SA in inducing oxidative stress in Arabidopsis seeds that are still in the germinating stage (Rampitsch1 & Bykova, 2012). Proteins that have been identified in roots are mostly associated with central metabolic pathways, transcription control and mRNA and protein biosynthesis (Park, 2004).

Mitochondrial proteomics have been well studied in *Arabidopsis thaliana* (Kruft *et al.*, 2001; Millar *et al.*, 2001). About 800 proteins were identified in *Arabidopsis thaliana* using proteomic approaches such as 2DE combined with mass spectrometry. Proteins that were identified are involved in respiration, citric acid cycle, amino acid and nucleotide metabolism, protein transport and antioxidant defence (Park, 2004).

The chloroplasts encode about 120 proteins of which most of them are transported from the nuclear membrane (Keegstra & Cline, 1999). The proteomic studies of *Arabidopsis thaliana*

chloroplast envelop membrane (Ferro *et al*., 2003), have shown identification of 100 proteins by LC-MS/MS analyses. Some of these proteins were involved in proteolysis, carbon metabolism and oxidative stress response. Secreted proteins also referred to as secretome (Greenbaum *et al*., 2001; Hathout, 2007) form an important component of the extracellular matrix of living cells (Showalter, 1993). The secretome and other components of the extracellular matrix play important roles in cell-cell interactions, growth and development, as well as defence mechanisms upon wounding or pathogen attack (Brownlee, 2002; Knox, 1995; Pennell, 1998; Showalter, 1993).

4.2 MATERIALS AND METHODS

4.2.1. PLANT MATERIAL CULTIVATION

Tugela DN and Tugela seeds were germinated cultivated and stressed and leaves harvested as described in Section 3.2.1.

4.2.2. EXTRACTION OF PHOSPHOPROTEINS

Proteins were extracted separately from 24 different plants using the phosphoprotein enrichment kit (Pierce) following the manufacturer′s instructions. In summary, 500 mg of Tugela DN or Tugela leaves were finely ground in liquid nitrogen to a fine powder. Cells were lysed in 1ml of Lysis/Binding/Wash buffer containing CHAPS, and 10 µl of 1xHalt protease inhibitor EDTA-free (Thermo Scientific) and 1xHalt Phosphatase inhibitor cocktail (Thermo Scientific). Cells were then incubated on ice for 45 minutes, with periodic mixing. They were then centrifuged at 10,000x *g* for 20 minutes at 4ºC to pellet cell debris. The protein concentration of the supernatant was determined using the micro BCA protein assay (Pierce) as described in section 3.2.3. The concentration was adjusted with the Lysis/Binding/Wash buffer to a concentration of 0.5 mg/ml.

Columns were equilibrated prior to loading of protein. Columns were inverted, bottom snapped off, the cap loosened, placed in a 50 ml conical tube and centrifuged at 1,000x *g* at 4ºC for 1 minute to remove the storage solution. The resin was equilibrated by adding 5 ml of Lysis/ Binding/ Wash buffer with CHAPS, placed in a 50 ml conical tube and centrifuged at 1,000x *g* at 4ºC for a minute. Diluted lysates (protein < 4 mg) were added to a plugged column, capped, inverted several times, centrifuged and placed on a platform rocker for 30 minutes at 4ºC. Bottom plugs were removed and the columns were placed in 50ml conical

tubes and were centrifuged at 1,000x *g* for 1 minute at 4ºC. The resin was washed with 5ml Lysis/Binding/Wash buffer with CHAPS, centrifuged at 1,000x *g* for a minute at 4ºC. The wash step was repeated twice for a total of three washes. The bottom of the columns were plugged, transferred into clean 50 ml conical tubes, and 1ml of elution buffer without CHAPS was added to each column and the columns were incubated at room temperature with occasional agitation for 3 minutes. Plugs were removed and columns were centrifuged at 1, 000x *g* for a minute at 4ºC. This step was repeated four times for a total of five elutions (5 ml). Fractions were pooled together and stored at -80ºC.

To concentrate the pooled phosphoprotein fractions, 5 ml of each sample was placed into the upper sample chamber of a (phosphoprotein enrichment kit) concentrator (Pierce). The concentrators were capped and centrifuged in a fixed angle rotor at a speed of 6000x *g* at 4ºC. Samples were centrifuged until the retentate was about 150-200 µl volume. The concentrated protein (retentate) was removed promptly using a 200 µl pipette tip to gently aspirate concentrated sample from upper chamber.

4.2.3 2D-CLEANUP

In order to improve focusing, 2-D cleanup was performed using the 2-D clean up kit from Bio-Rad (USA) following the manufacturer′s instructions. Two hundred and fifty micrograms of each protein sample was transferred into a 1.5 ml microcentrifuge tube. Precipitating agent 1 (300 µl) was added to each protein sample and mixed well by vortex mixing. Samples were incubated on ice for 15 minutes. Then 300 µl precipitating agent 2 was added to the above mixture and mixed well by vortexing. Tubes were centrifuged at a maximum speed of 13,000x *g* for 5 minutes to form a tight pellet and the tubes removed promptly, so that the pellet does not disperse. Supernatants were removed and discarded using a micropipette tip. The tubes were recentrifuged at 13,000x *g* for 15-30 sec to collect any residual liquid, which was carefully removed. To each pellet, 40 µl of wash reagent 1 was added, centrifuged at a maximum speed of 13,000x g for 5 minutes, and the supernatant discarded. Ultrapure water (25 µl) was added to the pellet, vortexed for 20-30 sec and incubated at -20ºC for 1 hr. Wash reagent 2 (5 µl) was added, vortexed for 1 minute and the tubes incubated at -20ºC for 30 minutes, with periodical vortex mixing every 10 minutes. After the incubation, tubes were centrifuged at 13,000x *g* for 5 minutes to form a tight pellet and the supernatant discarded. Tubes were further centrifuged briefly and any residual wash

solution was discarded. Pellets were then air-dried at room temperature for a maximum of 5 minutes.

4.2.4. READY- PREP 2D- STARTER KIT

Pellets were re-suspended in 250 µl sample/ rehydration buffer (8 M urea, 2% CHAPS, 50 mM dithiothreitol (DTT), 0.2% (w/v) Bio-Lyte®, 3/20 ampholyte and bromophenol blue (trace) provided with 2D starter kit. The cover sheet of the IPG strips were peeled using forceps and the IPG strips (7cm; pH 3-10 non-linear gradient) rehydrated by placing them face down on 125 µl of protein sample which had been pipetted as a line on the bottom of each channel of the rehydration tray, avoiding introduction of air bubbles. Each of the strips was overlaid with 2ml of mineral oil to prevent evaporation of the samples. The rehydration chamber was covered with a plastic lid and the tray left on a level bench overnight.

Isoelectric focusing was performed, using a clean dry PROTEAN® IEF focusing tray, the same size as rehydrated IPG strips (7 cm). Paper wicks, presoaked with 8 µl of nanopure water, were placed with forceps on both ends of the channels to cover wire electrodes. The IPG strips were held vertically using forceps to allow the mineral oil to drain, and transferred to the corresponding channel in the focusing tray, with the gel side down. The strips were covered with 2 ml of fresh mineral oil (Bio-Rad), being careful not to trap any air bubbles and the tray was covered with a lid. The focusing tray was placed into a PROTEAN® IEF cell and the cell was programmed using the following 3-step protocol: step 1- 250 V for 20 minuts, in a linear ramp, step 2- 4000 V for 2 hrs, linear ramp and step 3- 4000 V for 10,000 V.hr, in a rapid ramp.

On completion of IEF, the IPG strips were removed from the tray and incubated (with shaking) in 2.5 ml of equilibration buffer I (6M urea, 2% SDS, 0.375 M Tris-HCl (pH 8.8), 20% glycerol and 2% (w/v) DTT). Followed by incubation in equilibration II (6 M urea, 2% SDS, 0.375 M Tris-HCl (pH 8.8) and 20% glycerol with 0.5 g iodoacetamide, and 30% glycerol (13.35 ml). After the equilibration steps the IPG strips were briefly rinsed in 100 ml of 1xTris-Glycine-SDS running buffer. The strips were then transferred onto the top of a Mini-PROTEAN TGX Gels, (7cm IPG strip, 10% "Any kD "resolving gel) for the second dimension. A Pasteur pipette was used to overlay malten agarose solution (BioRad) on top of the IPG strip. The second dimension gels were run at a constant 200V for 45 minutes before

staining the gel with Coomassie Brilliant Blue G-250 (Bio-Rad) followed by Silver Stain Plus (Bio-Rad).

4.2.5 IDENTIFICATION OF GEL SPOTS BY MASS SPECTROMETRY

Spots were identified in each gel, cut out using a sterile scalpel and placed in a 1.5 ml tube. The gel pieces were covered with acetonitrile, incubated for 5 minutes before removing the acetonitrile and drying in a Speed Vac. Dried gel pieces were labeled and sent to the Centre for Proteomic and Genomic Research (CPGR) for Mass Spectrometer analysis to identify the proteins in the cut out, dried gel pieces.

In gel digest

All reagents are analytical grade or equivalent. Gel slices supplied were destained in an Eppendorf 1.5 ml tube with 200 mM NH_4HCO_3: Acetonitrile 50:50 (Burdick & Jackson; Sigma) until clear. Samples were dehydrated and desiccated before reduction with 2 mM triscarboxyethyl phosphine (TCEP; Fluka) in 25 mM NH_4HCO_3 for 15 minutes at room temperature with agitation. Excess TCEP were removed and the gel pieces again dehydrated. Cystein residues were carbamidomethylated with 20 mM iodoacetamide (Sigma) in 25 mM NH_4HCO_3 for 30 minutes at room temperature in the dark. After carbamidomethylation the gel pieces were dehydrated and washed with 25 mM NH_4HCO_3 followed by another dehydration step. Proteins were digested by rehydrating the gel pieces in trypsin (Promega) solution (20 ng/ul) and incubating at 37°C overnight. Peptides were extracted from the gel pieces once with 50 µl 0.1% trifluoroacetic acid (TFA) (Sigma). The samples were dried down and 200µl water added and concentrated to less than 20 µl to remove residual NH_4HCO_3

The samples were dried and re-dissolved in 0.1% TFA. The samples were purified and concentrated using a C_{18} ZipTip® according to the manufacturer′s instructions. The purified samples were eluted with α-cyano-4-hydroxycinamic acid (CHCA) at 5 mg/ml in 50% acetonitrile/H_2O containing 0.1% trifluoroacetic acid (TFA) and spotted manually onto a MALDI target plate.

Mass spectrometry

MALDI MS was performed using a 4800 MALDI ToF/ToF system (AB SCIEX) with instrument control through 4000 Series Explorer. Parent spectra were acquired in reflector positive mode at a laser intensity of 4000 arbitrary units using 600 laser shots per spectrum. The scan range was m/z = 800 - 4000. The grid voltage was set to 16 kV. Spectra were internally calibrated using trypsin autolytic fragments. Fragmentation data was acquired in positive mode with a deceleration voltage of 1 kV. These spectra were acquired with a laser intensity of 4500 arbitrary units and 1600 shots per spectrum.

Data Analysis

Database interrogation was performed with the Mascot algorithm using the MSDB database on a GPS workstation. Search parameters were as follows:

Species – All; Plant

Enzyme – trypsin;

Maximum number of missed cleavages -1;

Fixed modifications – carbamidomethyl (C);

Variable modifications oxidation (M); deamination (N/Q); pyroglutamic acid

Precursor tolerance - 100 ppm.

Fragment tolerance – 0.2 Da

4.3 RESULTS AND DISCUSSION

Wheat seeds (Tugela DN and Tugela) were all germinated in Petri dishes on moist Whatman filter paper and grown in a controlled environment chamber. Seedlings were then planted in pots containing a mixture of clay soil, sand and vermiculite. Both the Tugela DN and the Tugela plants were infested with RWA, with appropriate controls (uninfested plants) for comparison. Cultivation of Tugela DN wheat under aphid infestation lead to visible chlorosis after 120 hrs of the aphid stress as compared to the Tugela plants where chlorosis was visible after 40 hrs. (Fig 4.1 B).

A B

Figure 4.1: Aphid infested tugela Dn and Tugela wheat plants grown under controlled environmental conditions in a conviron S1OH manufactured by Controlled Environments Ltd., Winnipeg, Canada, under the following conditions: temperature 25^{o}C, humidity 56%, CO_2 (ambient) and a light intensity of 400µmol $m^{-1}sec^{-1}$. Infestation was achieved by transferring 10 aphids onto a single leaf per plant, within a leaf cage.

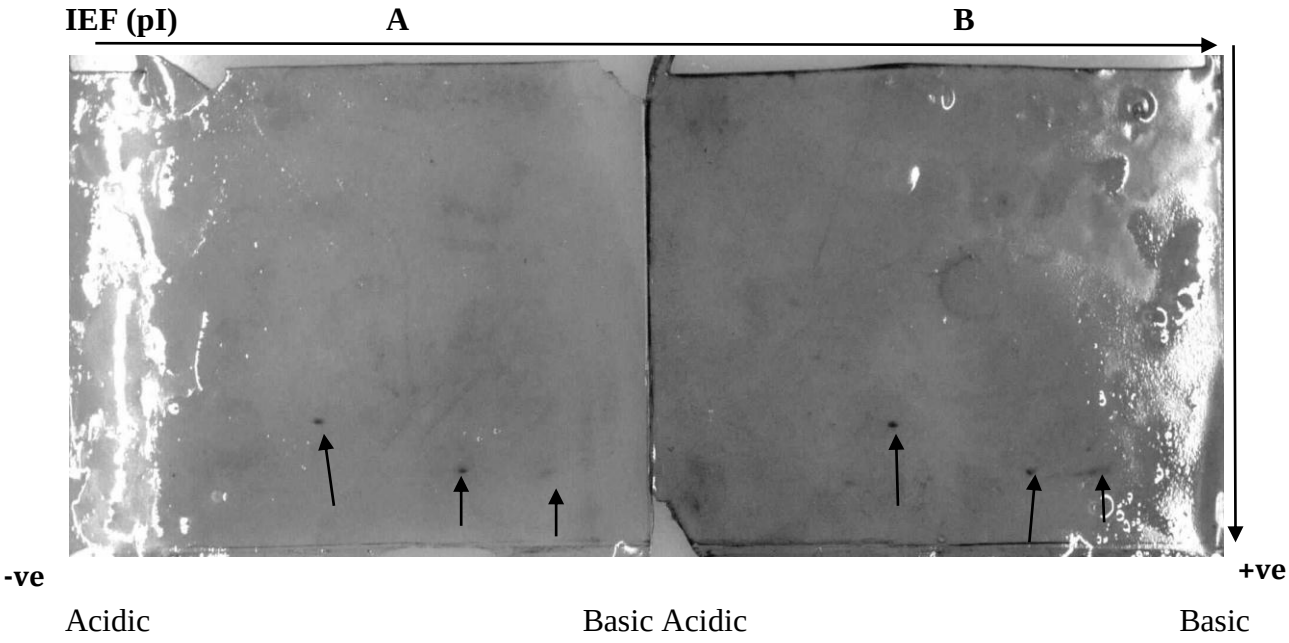

Figure 4.2: Detection of aphid infestation wheat response proteins by 2D Gel Electrophoresis. Tugela DN and Tugela seeds were germinated and planted in pot plants for up to 216 hours with harvesting of the leaves at 0, 1, 3, 5 ,7 and 9 days. The above figure represents phospho-enriched soluble proteins in Tugela (A) and Tugela DN (B) leaves after 48 hours infestation.

Sixty phosphoprotein spots (some gels not shown) were identified by 2D gels stained with Coomassie Blue R250 and silver stain. These gels were then visualized and photographed using Alliance 4.7 Transilluminator (UVITEC Ltd, Cambridge, UK). However, only three spots were identified with high confidence (highlighted in bold in Table 4.1). Several other spots revealed protein sequences that only partially matched protein sequences in the queried protein sequence databases, and since the percentage match was below the accepted limit, these matches where labeled as 0% confidence interval.

Table 4. 1: Proteins identified with positive identifications in bold. Protein significance level is: protein score ≥ 67. Phosphoproteins were ex*cis*ed from Coomassie Blue R250/Silver stained gels, destained, trypsin digested and identified by MALDI MS using a 4800 MALDI ToF/ToF system (AB SCIEX). Fingerprints obtained were matched to protein sequences in the MSDB using the Mascot software on a GPS workstation. Sample identification number refers to Tugela (**T**), Tugela DN (**D**), Stressed (**S**), Unstressed (**U**); first number refers to time of infestation and second number to the spot number on the relevant gel e.g. TS-9-5 refers to spot five on gel of sample leaf from Tugela wheat after 9 days infestation with aphids.

Spot Index	Sample identification number	Protein identified	Protein score	% confidence interval
A2	**Control**	**BOVALBUMIN NID: - Bos Taurus**	**476**	**100**
A17	β -TS-0-1	Hypothetical protein rps12 - Oenothera hookeri (Hooker′s evening primrose).	15	0
A18	β -TS-0-2	Putative SNARE protein - Hevea brasiliensis	24	0
A8	β -TS-1-1	ACC synthase homolog (Fragment) - Rumex palustris	34	0
A9	β -TS-1-2	Bowman-Birk type proteinase inhibitor PVI-4 - Phaseolus vulgaris	12	0
A6	β -TS-1-3	AY131259 NID: - Pseudotsuga menziesii	36	0
A10	β -TS-1-4	Leunig (Fragment) - Brassica rapa subsp. Pekinensis	30	0
A11	β -TS-1-5	CQ817684 NID: - Bromus	23	0
A7	β -TS-1-6	Reverse transcriptase (Fragment) - Medicago sativa (Alfalfa).	33	0
A12	β -TS-1-7	Histone H1 - Pisum sativum	51	33.892
A13	β -TS-1-8	Polygalacturonase inhibitor protein (Fragment) - Mirabilis jalapa	32	0
A14	β -TS-1-8a	PsbJ (Fragment) - Ipomoea quamoclit	11	0
A15	β -TS-3-1	MIBTRPS NID - Oenothera berteriana	14	0
A16	β -TS-3-2	Polygalacturonase inhibitor protein (Fragment) - Mirabilis jalapa	24	0
A5	β -TS-9-1	Sequence 5 from Patent WO0164900 (Fragment).- Physcomitrella patens (Moss). Keratin 10, type I, cytoskeletal - human	40 67	0 62.978
A19	**β -TS-9-2**	**Lea protein – soybean** **HSKERAT9 NID - Homo sapiens**	**38** **152**	**0** **100**
A20	β -TS-9-3	Hypothetical protein A - Bertero′s evening primrose mitochondrion	18	0
A21	β-TS-9-4	Gibberellin 3-oxidase - Spinacia oleracea	27	0

A4	**β -TS-9-5**	**Photosystem II oxygen-evolving complex protein 2 precursor – wheat**	**59**	**89.279**
A22	β-TU-1-1	Generative cell-specific histone H3 - Lilium longiflorum	30	0
A23	β-TU-3-1	Histone H1 - Pisum sativum	35	0
A24	β-TU-3-2	AF494278 NID - Chaetosphaeridium globosum	41	0
B1	β-TU-3-3	Hypothetical chloroplast RF66 - Huperzia lucidula	32	0
B2	β-TU-3-4	S)-2-hydroxy-acid oxidase (EC 1.1.3.15) (with FMN and an inhibitor), fragment 2 - spinach	34	0
B3	β-TU-3-5	Phosphoenolpyruvate carboxylase (Fragment) - Gaertnera junghuhniana.	32	0
B4	β-TU-3-6	AY049012 NID - Chenopodium album	41	0
B5	β-TU-3-7	Histone H2B – pepper	21	0
B6	β-TU-3-8	Ribonuclease H (Fragment) - Phaseolus vulgaris	16	0
B7	β-TU-5-1	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (Fragment) - Bletia purpurea.	18	0
C3	α-DS-0-1	Putative late blight resistance protein - Solanum demissum	47	0
C4	α-DS-0-2	Cyclin A-type (clone 13) - common tobacco (fragment)	26	0
C5	α-DS-0-3	R2R3MYB-domain protein (Fragment) - Zea mays (Maize).	25	0
C6	α-DS-0-4	No protein found		
C7	α-DS-1-1	sfti-1, chain I - common sunflower	26	0
C8	α-DS-1-2	Hypothetical protein - Nephroselmis olivacea.	32	0
C9	α-DS-1-3	Hypothetical protein 43d - Japanese black pine chloroplast	7	0
C10	α-DS-5-1	AF037457 NID - Fritillaria agrestis	28	0
C11	α-DS-5-2	Putative polyprotein - Nicotiana tabacum (Common tobacco).	18	0
C12	α-DS-7-1	Small ribosomal protein 4 (Fragment) - Cunninghamia lanceolata	13	0
C13	α-DS-9-1	AF037457 NID - Fritillaria agrestis	40	0
C14	α-DS-9-2	Farnesyl-pyrophosphate synthetase - Artemisia annua	52	46.266
C15	α-DS-9-3	EF-1 alpha (Fragment) - Acetabularia acetabulum	23	0
C16	α-DS-9-4	Photosystem II oxygen-evolving complex protein 2 precursor – wheat	41	0
C17	**α-DS-9-5**	**HVUNKNOWN NID - Hordeum vulgare subsp. Vulgare**	**65**	**97.487**

C19	α-DU-0-1	Photosystem II oxygen-evolving complex protein 1 -common wheat x Sanduri wheat	35	0
C20	α-DU-0-2	Phytochrome (Fragment) - Torreya nucifera.	18	0
C21	α-DU-0-3	R2R3MYB-domain protein (Fragment) - Zea mays (Maize).	28	0
C22	α-DU-3-1	Putative polyprotein - Nicotiana tabacum (Common tobacco).	16	0
C23	α-DU-5-1	Histone H2B.2 – wheat	45	0
C24	α-DU-5-2	Putative polyprotein - Nicotiana tabacum	17	0
D1	α-DU-5-3	Transposase (Fragment) - Arundinaria simonii.	30	0
D2	α-DU-7-1	AF037457 NID - Fritillaria agrestis	45	0
D3	α-DU-7-2	Phytochrome (Fragment) - Torreya nucifera.	22	0
D4	α-DU-7-3	Photosystem II oxygen-evolving complex protein 2 precursor – wheat	19	0
D5	α-DU-7-4	NBS-LRR resistance gene-like protein ARGH11 (Fragment) - Malus domestica	16	0
D6	α-DU-9-2	Profilin (Fragment) - Capsicum annuum	12	0
D7	α-DU-9-3	Trypsin inhibitor precursor (clone ATI18) - alfalfa	21	0
D8	α-DU-9-4	Pathogenesis-related protein - chickpea	12	0
D9	α-DU-9-5	AtpB (Fragment) - Agonis spathulata.	16	0
D10	α-DU-9-6	Putative fructokinase (Fragment) - Pinus sylvestris	9	0

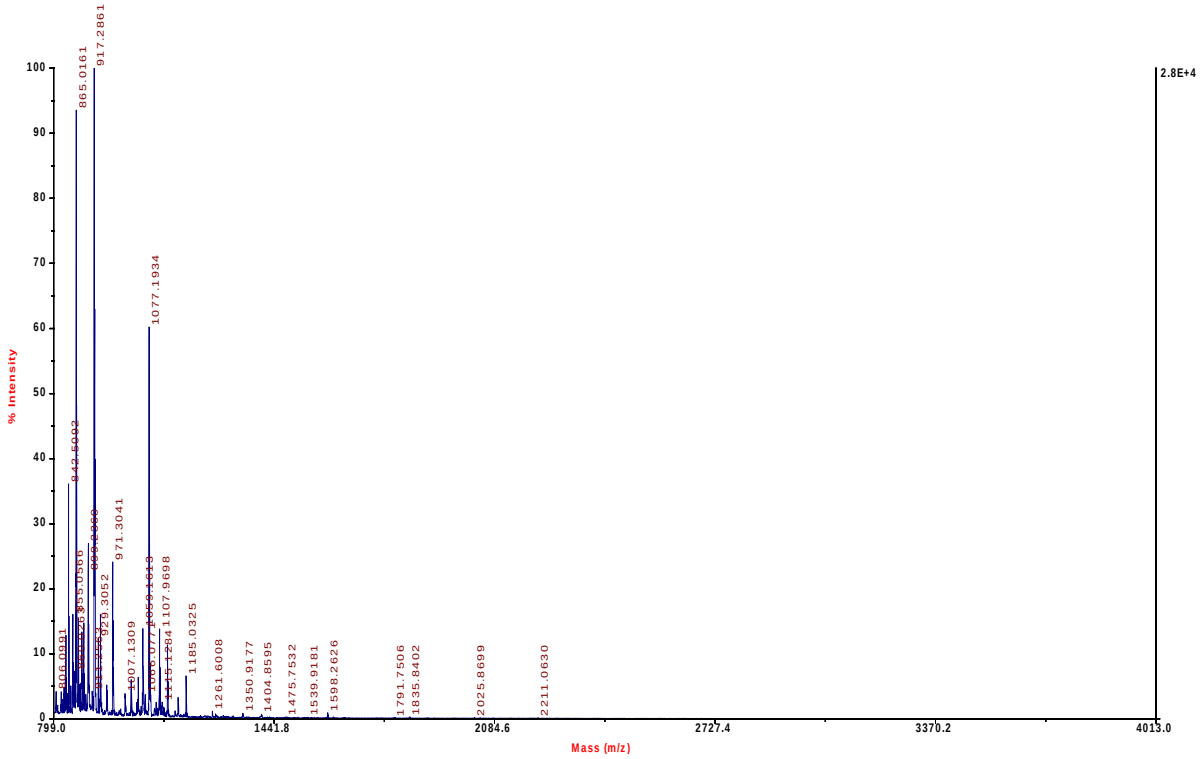

Figure 4.3: A spectra showing; α-DU-9-5- HVUNKNOWN NID: - Hordeum vulgare subsp. vulgare

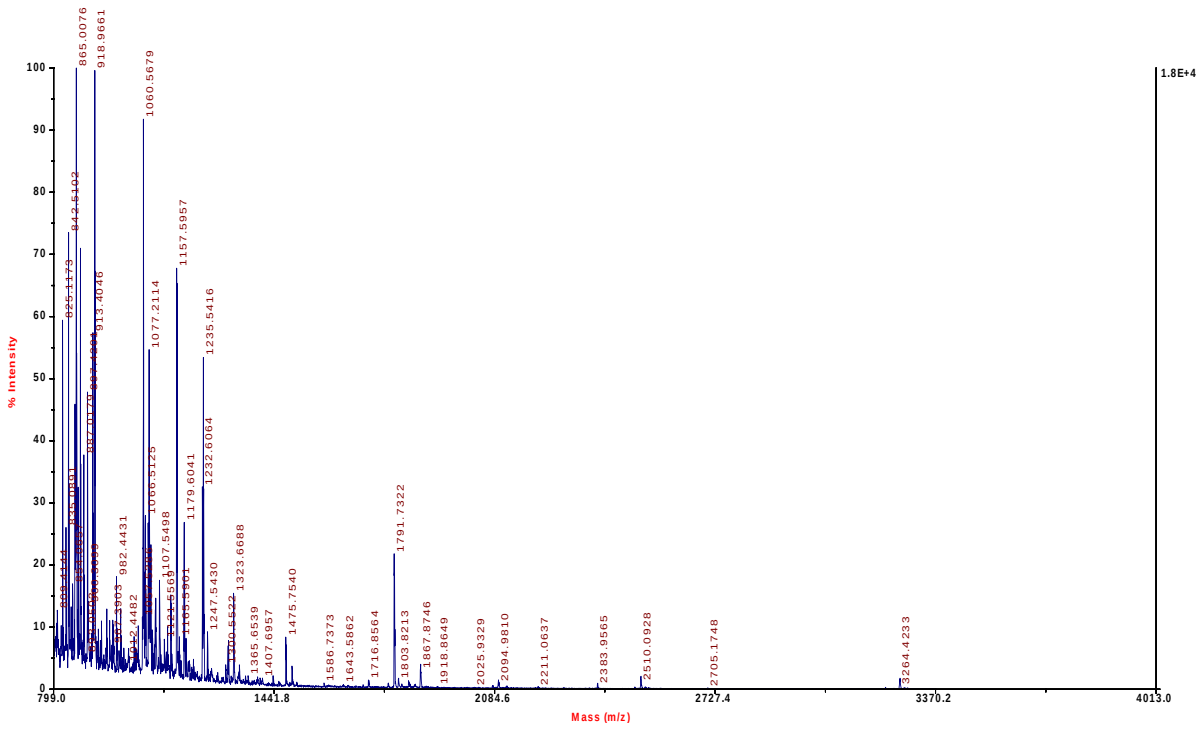

Figure 4 .4: A spectra showing β -TS-9-2-HSKERAT9 nid: - *Homo sapiens* which was positively in wheat

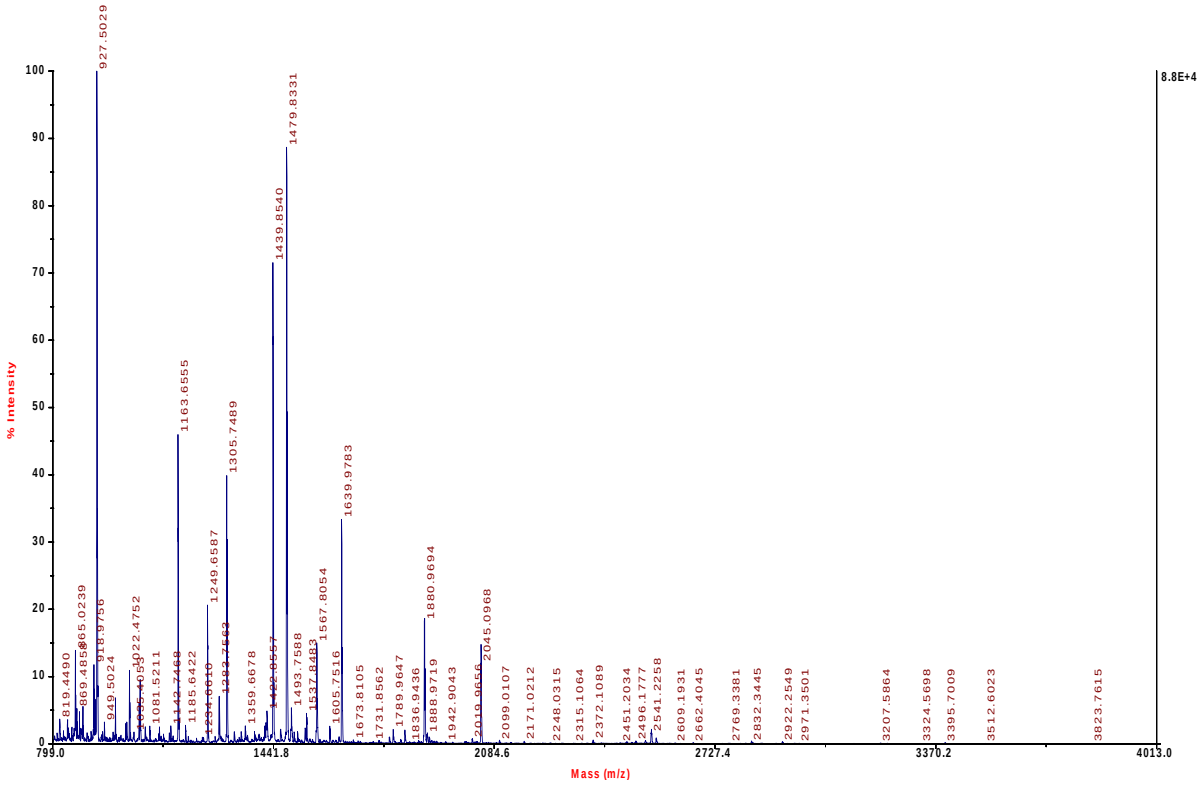

Figure 4.5: A spectra showing BSA that was used as a control

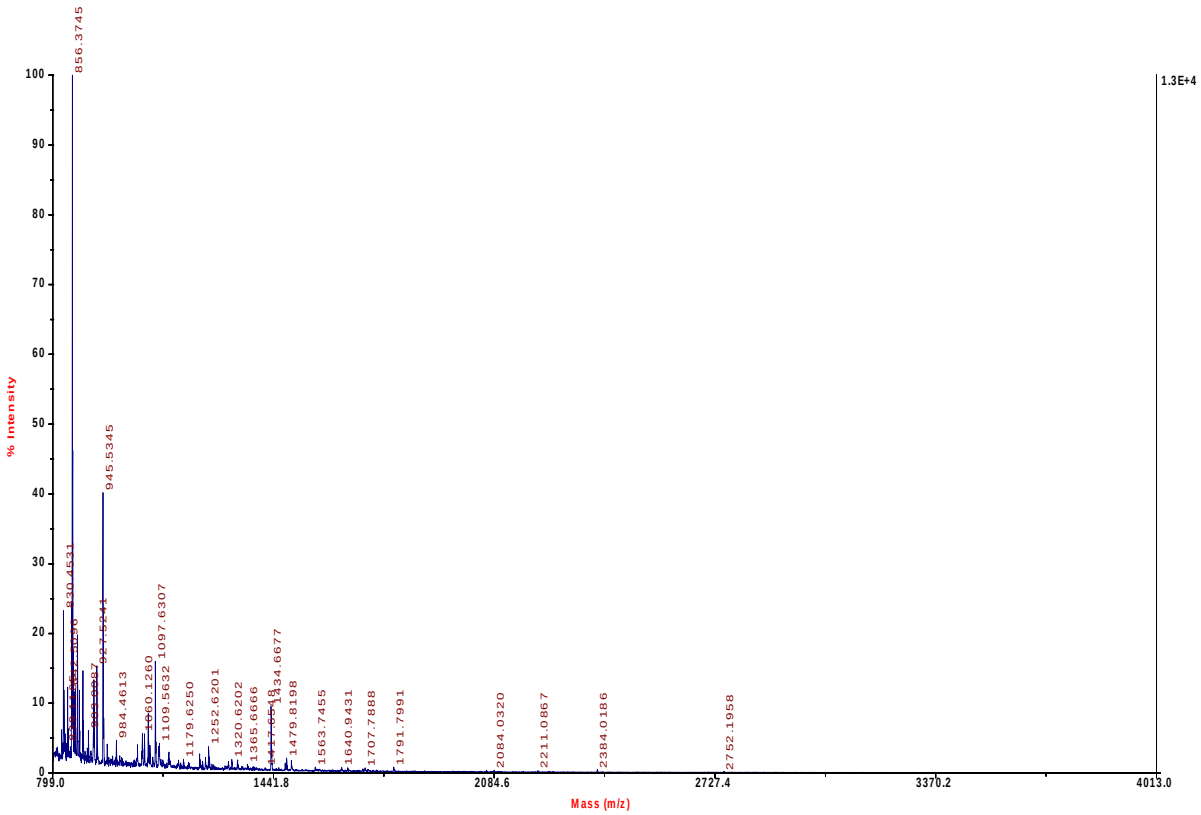

Figure 4.6: A spectra showing β -TS-9-5.-Photosystem II oxygen-evolving complex protein 2 precursor – wheat

Sixty (60) spots were ex*ci*sed from the twenty-four 2D gels, seventeen (17) from the unstressed Tugela DN; fifteen (15) from the stressed Tugela DN; ten (10) from the unstressed Tugela and eighteen (18) from the Tugela stressed. The ex*ci*sed proteins were submitted for in-gel digest and Peptide Mass Fingerprinting at CPGR (Cape Town). Only three (3) proteins were positively identified out of the fifty-nine protein spots. Fifty-seven (57) proteins could not be identified above the required confidence level. The process control, Bovine Serum Albumin, was positively identified.

The three proteins that were positively identified were all up-regulated in response to aphid infestation, two in the Tugela susceptible strain (β-TS-9-2-HSKERAT9 NID - Homo sapiens; β-TS-9-5 - Photosystem II oxygen-evolving complex protein 2 precursor – wheat) and one in the Tugela DN resistance strain (α-DU-9-5- HVUNKNOWN NID - Hordeum vulgare subsp. Vulgare) (Table 4.1) all after 216 hrs of aphid infestation. The Photosystem II oxygen evolving complex protein 2 precursor can be linked to a possible role in stress by preventing chlorosis by sustaining photosynthesis (Figure 4.7).

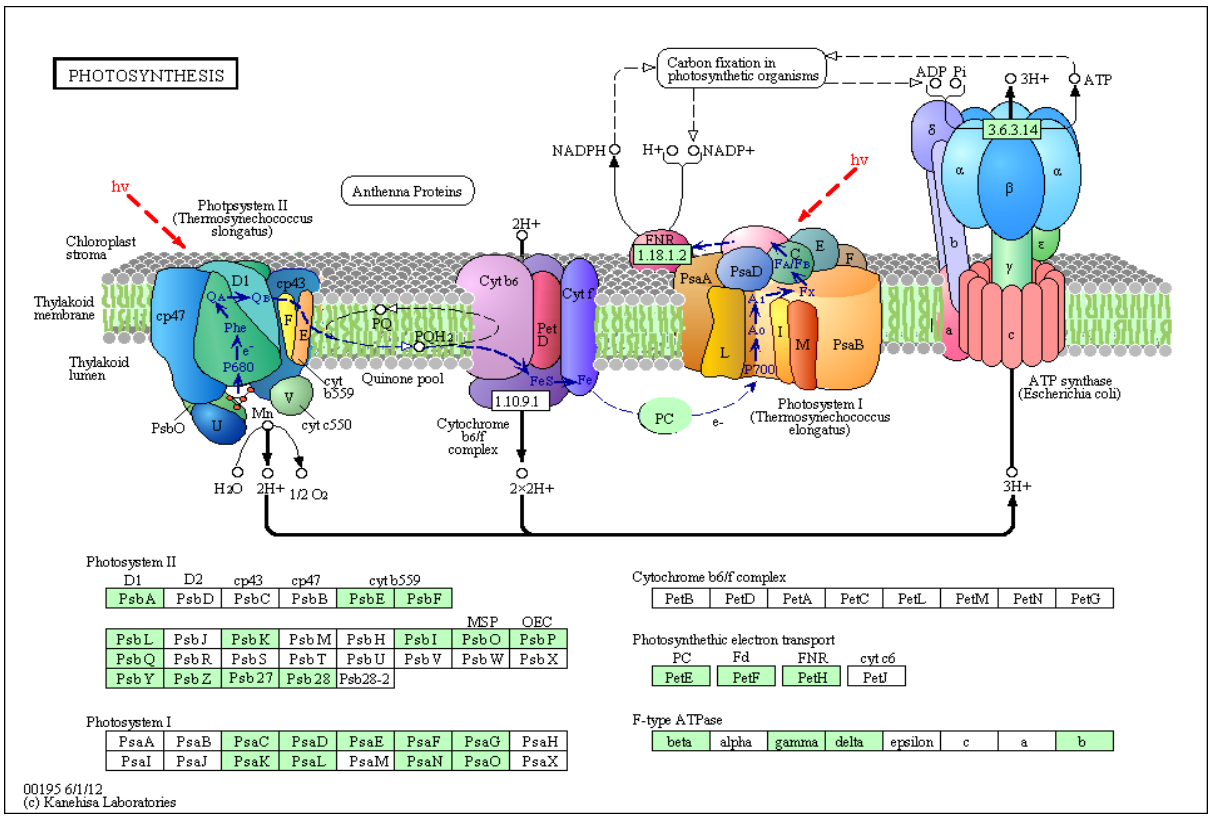

Figure 4.7: Photosynthesis pathways from Oryza sativa japonica (Japanese rice). Taken from KEGG (http://www.genome.jp/dbget-bin/www_bget?dosa00195) – Pathway dosa00195.

The “HSKERAT9 NID - Homo sapiens protein” (CAA82315) is a cytokeratin 9 protein in *Homo sapiens*. This protein has a sequence similarity to a protein found in rice, viz. Glycine-rich cell wall structural protein 2 (P0C5C7), which is known to be induced by viruses (Fang *et al*., (1991). However, keratin is a common contamination found in 2-D gel analysis, due to protein contamination from fingers or hair from the operator during sample or gel handling.

No function could be ascribed to the “HVUNKNOWN NID - *Hordeum vulgare* subsp. vulgare” protein.

The fifty-seven (57) samples that could not be identified with a high degree of confidence, were in our opinion low abundance proteins and therefore due to the low protein concentrations in our samples resulted in too few matches in the finger print identification process. However, when one analyses the possible identities of those proteins, it is interesting to note that several of them are stress related proteins.

Three of the four stress related proteins found in Tugela infested plants were inhibitors, Bowman-Birk type proteinase inhibitor PVI-4 -Phaseolus vulgaris (A9: β-TS-1-2); Polygalacturonase inhibitor protein (Fragment)-Mirabilis jalapa (A13: β-TS-1-8); Polygalacturonase inhibitor protein (Fragment)-Mirabilis jalapa (A16: β -TS-3-2).

One of the two stress related proteins from the Tugela DN infested plants is a “Putative late blight resistance protein - *Solanum demissum*” (C3: α-DS-0-1), with the other identified as a Photosystem II oxygen-evolving complex protein 2 precursor – wheat (C16: α-DS-9-4), the same as one of the positively identified as up-regulated in the infested Tugela susceptible plants (β-TS-9-5).

Three of the five proteins from the uninfested Tugela DN resistance plants were identified as stress or pathogen proteins, NBS-LRR resistance gene-like protein ARGH11 (Fragment) - Malus domestica (D5: α-DU-7-4); Trypsin inhibitor precursor (clone ATI18) – alfalfa (D7: α-DU-9-3); Pathogenesis-related protein – chickpea (D8: α-DU-9-4). The other two proteins were identified as Photosystem II oxygen-evolving complex protein, again the same protein positively identified as up-regulated in the infested Tugela susceptible plants (β-TS-9-5).

4.4. Conclusion

The objective of this study was to identify possible signalling pathways induced in cereals by phloem feeding through profiling the phosphoproteome of wheat during infestation by Russian wheat (*Diuraphis noxia*) aphid using 2DE. The study was partially successful in that phosphoproteins were isolated by using the phosphoproteome enrichment kit together with phosphate inhibitors to prevent dephosphorylation. However, only three proteins could be positively identified, with two of these found only in the Tugela susceptible plants. The other fifty-seven proteins could not be conclusively identified, due to the low percentage of peptide matches to known proteins. It is possible that the reason these could not be conclusively identified is because they are low abundance proteins and therefore the level of protein in the 2-D gels was insufficient for MS analysis. Future studies will therefore have to include prefractionation of the proteins to remove high abundant proteins as well as running multiple 2D gels of the same extracts so that the same spots can be pooled to increase their concentration prior to MS analysis. 2D gel electrophoresis was therefore not as successful as LC/MS analysis, in identifying proteins involved in the stress or resistance responses of wheat.

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Chapter 5

Discussion and summary

Wheat is an important staple food in South Africa; second only to maize in production levels, therefore understanding factors that may affect the yield of wheat crops is vital for food security in SA. Wheat production has been challenged by a multitude of biotic and abiotic stresses. RWA infestation has been reported to cause yield losses of up to 90% per individual plant, thus leading to huge economical yield losses (Tolmay *et al*., 2007). RWA resistant cultivars were first produced in South Africa shortly after the discovery of host plant resistance to RWA by bread wheat (Du Toit, 1987; 1988; 1992). The first resistant cultivar, Tugela DN was discovered in 1992 and by 2006, 27 cultivars had been developed (Tolmay *et al*., 2007). Tugela DN and Betta DN are well known resistant wheat cultivars containing the *Dn1*-resistant gene which is only effective against the RWA biotype (RWASA1) (Tolmay *et al*., 2007). The RWASA2 aphid strain, recently discovered in South Africa, has caused severe damage in commercial resistant wheat cultivars containing the *Dn1*-resistance gene (Tolmay *et al*., 2007; Tolmay, 2006). In this study there was a notisable chlorosis to Tugela DN and Tugela leaves infested by RWA from day 5 of the infestation. This supports the idea that chlorosis and leaf roll are the most common visble symptom to plant infestion by RWA (Burd et al., 1993).Understanding the resistance mechanism of the DN resistant cultivars is crucial to the development of new resistant cultivars. Proteomics is the approach of choice to identify proteins differentially regulated in resistant cultivars is response to aphid infestation. Recent technology developments have broadened the tools available to protein chemists. This study evaluated three different proteomic approaches, including Bioinformatics, LC/MS/MS and 2D gel electrophoresis, to identify the differentially regulated proteins and phosphoproteins involved in the DN resistance mechanism.

Callose deposition has been linked to aphid feeding and therefore callose synthases were selected as a model protein family to evaluate the bioinformatics approach in identifying proteins involved in the resistant response of cereal crops. Rice was used in this bioinformatic study as its genome has been completely sequenced. Ten rice and twelve Arabidopsis callose synthase genes were identified. The 2000 bp 5′ upstream region of the coding region for each callose synthase was analyzed for the presence of *cis*-regulatory

elements. The majority of the known *cis*-regulatory elements identified are involved in drought, light and extreme temperature responses. Only one *cis*-element involved in the wound or pathogenesis response was identified. A major short coming of this approach is that it can only be used on proteins already suspected to be involved in the resistance response and cannot be used to identify as yet unidentified stress proteins. This approach could possibly be used in the future when bioinformatics tools are developed that can scan whole genomes for *cis*-regulatory elements and identifying the proteins encoded by the downstream coding region of regions rich in specific *cis*-elements of interest, such as those involved in the wound response or pathogenesis response. Another shortcoming of this approach is that only those organisms for which the whole genome sequence is known can be studied.

Recent technological developments have improved the sensitivity of mass spectrometers and reduced the amount of protein needed for analysis, through nanotechnology, thereby making LC/MS systems available for high-throughput proteomics research. An Applied Biosystems QTRAP 4000 hybrid ion-trap mass spectrometer fitted with an Agilent 1200 Nanoflow High Performance Liquid Chromatography system was used to identify differentially regulated total proteins as well as phosphoproteins involved in signalling pathways activated by the aphid infestation of wheat.

A study by Scott *et al*. (2013) reported the identification of cytochrome oxidase B, hydroxymethylglutaryl-CoA lyase, cofilin, putative Di-Ras GTP binding protein, chitooligosaccharidolytic β-Nacetyl, acetylglucosaminyltransferase, hydroxymethylglutaryl CoA lyase, calreticulin, ficolin, aminopeptidase, acyltransferase, glucose dehydrogenases in wheat infested with RWA using the EST database. However in this study only dehydrogenase were identified.

Eight proteins were identified as involved in the Tugela DN stress response to aphid infestation including Putative mitochondrial cysteine synthase (B7TQG1), putative ascorbate peroxidase (Q8GZB9), putative uncharacterized proteins (A2WKE2, A2X3G6, A2YMU2 , A2XNY1, and A2ZGB9), fructokinase (A2WXV8) and ribosome-recycling factor. These are mostly involved in metabolic pathways, indicating a higher need for nutrient or energy in plants trying to recover from the effects of cell damage inflicted by aphid feeding.

$P \leq 0.10$ were cellular retinaldehyde-binding protein, trehalase, two separate,

Eleven proteins were identified as involved in the Tugela DN resistance mechanism, including aldehyde dehydrogenase family 7 members A1 (B6TB11), putative uncharacterized protein (B6TD97), putative mitochondrial cysteine synthase (B7TQG1), putative uncharacterized protein (B8AGD0), Photosystem I reaction center subunit VI, chloroplastic (P20143), Ribulose bisphosphate carboxylase/oxygenase activase B, chloroplastic (Q42450), putative uncharacterised protein (A2XNY1), Ribosome-recycling factor(A2ZBG9), chloroplastic (A2YMU2), putative uncharacterized protein (A2YMZ1), putative uncharacterized protein (A2YMZ1) and 40S ribosomal protein S26 (B4FEE7). Many of these proteins are involved in maintaining the integrity of the chloroplasts and the photosynthetic systems, suggesting that maintenance of photosynthesis is a key step in the resistance mechanism of Tugela DN plants.

Seven phosphoproteins, ATP synthase subunit alpha (Q6ENH7), ATP synthase subunit alpha (P0C2Z6), ATP synthase subunit beta (Q6ENG7), Ferredoxin NADP reductase leaf isoenzyme (P141344), Ferredoxin-nitrite reductase/chloroplastic (Q42997), hypothetical protein (P14655), Peroxiredoxin -2E -2 (Q7F855), photosystem II stability/ assembly factor HCF136 (Q5Z5A8), pyruvate / phosphatase dikinase (Q75KR1), zinc finger cccH domain-containing protein 20 (Q0DVU4), and zinc finger protein (Q9FDX8) were also identified by the LC-MS/MS system, as involved in Tugela DN resistance response to aphid infestation. The above mentioned phosphoproteomes are mainly involved in metabolic pathways, which again indicate that energy production is required in the stress response of wheat to aphid infestation.

Although a limited number of proteins and phosphoproteins were identified in this study, it was possible to differentiate between proteins involved in the stress response and those involved in the resistance response. An important factor in the success of this approach is identifying the correct time frames for protein sampling in order to capture the correct window period for the phosphorylation of proteins or up- or down-regulation of the proteins involved in the stress response or the resistance mechanism. Phosphoprotein enrichment technique was used to identify phosphoryted proteins. The precursor ion scan method used to identify the phosphorylated proteins had a limited success rate and therefore 2D gel electrophoresis was evaluated for the identification of phosphorylated proteins involved in the stress response or the resistance mechanism of cereals to aphid infestation.

Photosystem II oxygen-evolving complex protein 2 precursor**;** Sequence 5 from Patent WO0164900; keratin 10; cytoskeletal; AY131259 NID; Reverse transcriptase; ACC synthase homolog; Bowman-Birk type proteinase inhibitor PVI-4; Leunig; CQ817684 NID; Histone H1; Polygalacturonase inhibitor protein; PsbJ; MIBTRPS NID; Polygalacturonase inhibitor protein; Hypothetical protein rps12; Putative SNARE protein; Lea protein; Hypothetical protein A; Gibberellin 3-oxidase; Putative late blight resistance protein; Cyclin A-type (clone 13); R2R3MYB-domain protein; sfti-1; Hypothetical protein; Hypothetical protein 43d ; AF037457 NID; Putative polyprotein and Small ribosomal protein 4 were detected in 2D gels of phosphoproteins extracted from stressed Tugela and Tugela DN plants.

Generative cell-specific histone H3; Histone H1; AF494278 NID; Hypothetical chloroplast RF66; S)-2-hydroxy-acid oxidase; Phosphoenolpyruvate carboxylase; AY049012 NID; Histone H2B; Ribonuclease H; Ribulose-1,5-bisphosphate carboxylase/oxygenase and AF037457 NID; Farnesyl-pyrophosphate synthetase; EF-1 alpha; Photosystem II oxygen-evolving complex protein 2 precursor; Putative late blight resistance protein; Cyclin A-type (clone 13); R2R3MYB-domain protein; sfti-1; Hypothetical protein; Hypothetical protein 43d ; AF037457 NID; Putative polyprotein and Small ribosomal protein 4 were detected in unstressed Tugela and Tugela DN plants.

Although sixty protein spots were obtained in the 2D study, only three were identified with a high degree of certainty. The other fifty-seven proteins only partially matched known proteins, many of which are known to be stress or defence proteins, but the level of confidence of these matches were below acceptable threshold limits and therefore these proteins cannot be linked to either the stress or the resistance response of wheat to aphid infestation, but could serve as potential targets for future studies.

The objective of this study was to evaluate which proteomics approach would be the most suitable for identifying proteins and signalling pathways involved in cereal responses to aphid infestation. From the results obtained, it is therefore concluded that the LC/MS/MS was the most successful as it positively identified several proteins involved in either the stress or the resistance response of wheat to aphid infestation. It is also more versatile, in that it can identify differentially regulated proteins as well as phosphoproteins from the same sample without any additional sample preparations.

Future studies will have to include sub-fractionation of organelles, such as chloroplasts as well as shorter times of harvesting the leaves after infection, to identify earlier plant responses to aphid infestation.

5.1 References

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