Pyrenophora tritici-repentis ToxA requires Senescence Signals for the Induction of Necrotic Symptoms in *Triticum aestivum*

By

Jacqueline Ching

A thesis submitted to the Faculty of Graduate Studies in partial fulfillment of the requirements of the degree of

Master of Science

Department of Biochemistry and Medical Genetics

University of Manitoba

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

*****

COPYRIGHT PERMISSION

Pyrenophora tritici-repentis ToxA requires Senescence Signals for the Induction of Necrotic Symptoms in *Triticum aestivum*

BY

Jacqueline Ching

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirement of the degree

MASTER OF SCIENCE

Dedication

I would like to dedicate this thesis to my parents. To my Dad, who always pushes me to achieve more and to my Mom, who always makes me think I can.

Acknowledgements

I would like to thank my advisor Dr. Christof Rampitsch for his guidance, patience, and teachings, especially for the life lesson that "it'll all work out in the end," without your support I could not have persevered. I would like to thank my co-supervisor Dr. Gietz, for the great experience I had working with yeast in his lab last summer and for taking me on as a grad student in this faculty, without your initiative this degree would not have been possible. I would also like to thank my committee member Dr. Ballance, the scientist who discovered Ptr ToxA, without your discovery there would not be a project and thank you Dr. Jim Menzies for his general input and advice.

Thank you to Wayne Mauthe, a true master of the lab, and Eva Beimcik, the two 2DE specialists who helped me troubleshoot some pretty bad initial gels and supported me throughout the three years. Thank you to Dr. Bykova and Tao Fan for aiding me in the Mass Spec. analysis of the protein samples. Finally, I would like to thank all of my labmates, my friends and family for their general support, for listening to me complain when things didn't work and for cheering me on when they did, it was really appreciated, you guys kept me going and in a sense you all contributed a piece to this project.

Abstract

Pyrenophora tritici repentis is a fungus that produces Ptr ToxA, a host specific toxin that causes necrosis in ToxA-sensitive wheat cultivars. The molecular mechanisms that regulate this host pathogen interaction are unknown. In sensitive cultivars, toxin enters mesophyll cells and co-localizes with the chloroplast. Given the central role of chloroplasts in both programmed cell death and programmed senescence, it seems likely that chloroplasts initiate signals that induce necrosis. Leaves and isolated chloroplasts from Ptr ToxA sensitive and insensitive wheat cultivars were treated with purified Ptr ToxA and changes in their proteomes were evaluated by two dimensional gel elelctrophoresis. Affected proteins were selected and processed for analysis on a linear ion trap mass spectrometer and identified by Mascot. Proteins identified in ToxA sensitive and insensitive leaves suggest that the acceleration of senescence contributed to necrosis and identified proteins from the chloroplast of sensitive cultivars indicate that toxin perception initiates protective mechanisms.

Table of Contents

List of Tables

List of Figures

Abbreviation	Definition
Ado Met	Adenosyl methionine
2-DE	Two dimensional gel electrophoresis
ACC	1-aminocyclopropane-1-carboxylic acid
FNR	Ferredoxin NADP(H) oxido reductase
GS1	glutamine synthase 1
GS2	glutamine synthase 2
HPR	hydroxypyruvate reductase
HST	Host specific toxin
LHC II CAB	Light harvesting complex II Chlorophyll a/b binding protein
ROS	Reactive oxygen species
SDS-PAGE	Sodium dodecyl sulfate- polyacrylamide gel electrophoresis

Chapter 1: Introduction

1.1 Background

The genetics of plant pathogen interaction has been studied ever since it was noted that some combinations of pathogen isolate with host genotype resulted in different disease incidence or severity than others. In some plant pathogen relationships, such as rust, smuts and powdery mildew, the genetics of the interaction between these pathogens and their host has been well characterized. The knowledge gained from these studies has been useful in the development of pathogen resistant host cultivars. These studies have also led to a better understanding of how the pathogen and the host interact on a genetic level. Flor developed a gene for gene theory in an attempt to explain how pathogens such as the rusts, smuts, and powdery mildew genetically interact with their hosts (Flor, 1956). Flor's theory states "For each gene determining resistance in the host, there is a corresponding gene for avirulence in the parasite with which it specifically interacts". If this occurs it is deemed an incompatible reaction because the pathogen has an Avr (avirulent) gene factor that is recognized by the R(resistance)-gene factor, generally a receptor, in the plant which induces a host defense response. Compatible reactions are the result of the lack of presence of a R gene in the host, an avr gene in the pathogen, or non-recognition of either, which results in disease.

As more and different pathosystems were studied, different host pathogen interactions were discovered, including some which involved host specific toxins (HST). They were coined HST because the toxins produced by the fungal pathogen were very specific to the plants that serve as their hosts. The toxin is secreted by a fungus and acts

as the pathogenic factor. In the absence of toxin production, the pathogen is no longer virulent. Thus, these toxins are considered to be the causal agents of disease and compatibility (Walton, 1996) within the host. An incompatible/insensitive reaction in the host-pathogen system is the result of non-recognition of the toxin by the host or non toxin producing toxin, thus a reaction does not occur. In other words the toxin model is the inverse of the gene for gene model, where an incompatible reaction results from recognition that leads to an activation of defense signals. Generally when a compatible reaction occurs between a host specific toxin and its host, rapid cell death followed by necrosis is observed. The pathogen can then use the necrotic cells as food sources and spread through the host tissue.

Tan spot, a foliar disease in wheat caused by *Pyrenophora tritici repentis* (Died.) Drechs., anamorph *Drechslera tritici-repentis* (Died.) Shoem., is a pathogen that follows the HST model when it produces Ptr ToxA. Host sensitivity is conditioned by a single dominant gene located on chromosome 5BL (Faris *et al.*, 1996). This host specific toxin has been associated with pathogenicity (Tomas *et al.*, 1990, Ciuffetti *et al.*, 1997, Ballance *et al.* 1989) and sensitivity to this toxin has been associated with susceptibility (Lamari and Bernier, 1989b). Although many host specific toxins are low molecular weight metabolites, Ptr ToxA is a protein. It was first purified in 1989 by Ballance *et al.* and to date the structure, the host gene for compatibility, the mode of action and the resulting symptoms have been characterized. However, the molecular events involved in the expression of disease symptoms on the host have not been characterized fully.

1.2 Tan Spot

1.2.1 Disease significance

Tan spot is an economically significant foliar disease that has been reported worldwide. Yearly there are local and widespread cases of tan spot throughout most of the wheat producing nations, including North America, encompassing the Great Plains of the United States of America and the Canadian prairies. Tan Spot is a major disease of wheat in North America (Friesen *et al.*, 2002) that affects grain quality and production (Cuiffetti and Tuori, 1999; Lamari *et al.*, 2003). If symptoms are severe enough, foliar lesions can coalesce to form large areas of dead tissue (Lamari and Bernier, 1989 a and b). If this occurs on the flag leaf, it will hinder plant development or result in premature death. The loss of photosynthetic abilities of large areas of leaves as a result of tan spot lesions results in lower grain yields and test weights. Tan spot can result in yield losses ranging from 2- 50% (da Luz and Hosford, 1980, Rees and Platz, 1983).

Tan spot occurrence has increased since the 1970s (Strelkov and Lamari, 2003). This has been largely attributed to the shift from conventional to conservational or zero tillage practices (Suttons and Vyn, 1990), shorter rotations and continuous wheat cultivation (Rees *et al.*, 1982; Schilder and Bergstrom, 1995). The shift from conventional to zero tillage increases the presence of wheat stubble in fields allowing *Pyrenophora tritici-repentis* to over-winter and complete its life cycle so that it may infect the next generation of wheat.

1.2.2 Pathology of Tan Spot

Pyrenophora tritici-repentis has the ability to penetrate the epidermis and grow in the intracellular space between the mesophyll cells (Dushnicky *et al.*, 1996; Loughman and Deverell, 1986) where it releases toxins. A resistant reaction is characterized by a small brown to black fleck on the leaf (Lamari and Bernier, 1989) whereas a susceptible, or sensitive, reaction between the wheat leaves and the fungus produces a variety of characteristics. Lesions on the leaf may be chlorotic, necrotic or a combination of the two resulting in an irregular elliptical lesion with a brown necrotic center with surrounded by yellow chlorotic borders (Lamari and Bernier, 1989). The type of reaction is dependant on both the race of the isolate of fungus and the genotype of the wheat (Lamari and Bernier, 1989c, 1991). Thus different isolates of the fungus can induce different reactions in a single host plant (Ballance and Lamari, 1998, Lamari and Bernier, 1991) and a single race can induce different reactions in differential cultivars (Strelkov and Lamari, 2003).

Four main pathotypes have been identified that can cause different symptoms: pathotype 1 results in both necrosis and chlorosis, pathotype 2 results in necrosis; these are the two most common races in North America (Strelkov and Lamari, 2003). Pathotype 3 results in chlorosis, pathotype 4 causes neither. Although these pathotypes correspond to races 1-4 the two should not be confused, because races are also differentiated by size of lesions and effectiveness of infection on different hosts (Lamari *et al.*, 1995; Strelkov and Lamari, 2003). To date, eight races of *P. tritici repentis* have been

characterized and this number is predicted to increase as the differential sets expand and new isolates are tested (Strelkov and Lamari, 2003).

1.2.3 Sexual Cycle

Pyrenophora tritici-repentis reproduces by producing a black pin size fruiting sexual structure on wheat stubble that can survive the winter and in the spring these fruiting bodies release sexual ascospores and subsequently asexual conidia are generated throughout the season. These spores can be carried by the wind or rain splatter to neighbouring wheat seedlings. It is generally thought that ascospores are the initiating factors in tan spot epidemics, and that conidia serve to propagate the disease (Rees and Platz, 1983; Sutton and Vyn, 1990; Raymond *et al.* 1985). Wet humid conditions which favour large scale conidia production have lead to severe tan spot epidemics.

1.2.4 Methods of Prevention

To prevent spores from forming and reduce the level of disease at the early stages burying stubble through tillage has been used, but tillage increases the risk of soil erosion so is avoided if possible (Singh *et al.* 2006). Crop rotation is also used as a preventative measure but this must be managed carefully because it may cause the spread of other diseases for the next generation of wheat. Fungicides are also an effective, albeit costly, alternative. Some efforts have been focused on breeding and the development of genetically resistant wheat. Cultivars with high levels of resistance are available, however genotypes conferring complete resistance are rare (Rees and Platz, 1990). The development of improved genetically resistant wheat is the preferred method of disease

control because it is the most economical solution for the producer, and the safest for the environment (Zhang *et al.*, 1997). Genetic resistance is preferred over the other techniques because the resistance is inherited in the cultivar so no application methods are necessary. The development of such a product would be aided by an in-depth understanding of host pathogen interactions and the genes and the biochemical pathways involved in disease manifestation.

1.3 Ptr tox A

1.3.1 Host specific toxin

Two proteinacious host specific toxins from *P. tritici-repentis* have been identified; Ptr ToxB, a chlorosis inducing toxin (Strelkov *et al.*, 1998; Orolaza *et al.*, 1995), and Ptr ToxA, the necrosis inducing toxin. Ptr ToxA was the first toxin isolated from *P. tritici-repentis* and is the more extensively studied toxin. Ballance *et al.* (1989) isolated Ptr Tox A and demonstrated its role as a key factor in the development of necrosis in wheat which was shown by the following: all wheat lines that developed tan necrosis after infection with the fungus were also sensitive to the toxin; the resistant and chlorotic cultivars were all insensitive to the toxin; the toxin was capable of differentiating near isogenic lines differing in sensitivity; isolates from pathotypes that induced necrosis produced the toxin; and isolates lacking the ability to induce necrosis also lacked the ability to produce the toxin. The same dominant gene appears to be controlling development of necrotic symptoms, sensitivity to the toxin and disease susceptibility (Lamari and Bernier, 1989b). Therefore the toxin is necessary and sufficient to cause cell death in sensitive wheat cultivars.

Unlike other host specific toxins, this toxin is a protein (Ballance *et al*, 1989; Tomas *et al*, 1990; Tuori *et al.*, 1995) produced by a single gene at the *ToxA* locus. Ciuffetti *et al.* (1997) were able to show further its role as a pathogenic factor through transformation studies, whereby non-pathogenic isolates became pathogens when transformed with the ToxA gene.

1.3.2 Toxin Model

In both fungus and host, the factors for disease are governed by a single dominant gene in the Ptr-wheat pathosystem, and through conventional genetic analysis it appears that the development of symptoms induced by Ptr ToxA follows the toxin model. Sensitivity in wheat is conditioned by a single dominant gene, *Tsn 1*, located on the 5BL chromosome (Farris *et al.*, 1996), a gene rich recombination region (Haen *et al.*, 2004) and necrosis can only occur if both the *Tsn1* gene in wheat and *ToxA* gene in the fungus are present and this interaction follows the toxin model. The toxin model of Ptr ToxA is the inverse of the gene for gene interaction. In this system, recognition of the toxin by the host results in a compatible reaction, whereas in the gene for gene concept the recognition of Avr is an incompatible reaction. A compatible reaction in both systems means the pathogen can colonize the plants. With Ptr ToxA the recognition results in necrotic cell death which is beneficial for the fungus because necrosis facilitates fungal growth and propagation. In insensitive cultivars recognition of the toxin does not occur and necrosis is not induced, therefore the fungus can not infiltrate and spread. Recognition is speculated to be governed by a receptor since incompatibility is a receptor mediated response in fungal interactions that follow the gene for gene concept. To date,

a receptor mediating this even has not been discovered, but the toxin has been well characterized.

1.3.3 Primary Protein Structure

Ptr Tox A is a low molecular weight protein produced by the gene *ToxA* in *P. tritici-repentis*, which encodes for a pre-pro-protein (Ballance *et al.*, 1996, Ciuffetti *et al.*, 1997). The pre-pro-protein is comprised of a 178 amino acids, a pre region, residue 1 to 22, a pro-region, residue 23-60, and the mature protein, residue 61-178. The pre-region is proposed to contain a signal peptide that targets the protein to the secretory pathway (Ciuffetti *et al.*, 1997) and the pro region is cleaved prior to secretion and is speculated to be required for proper folding (Tuori *et al.* 2000) of the mature protein in the host. This 13.2 kDa toxin is comprised of a unique sequence without any potential structural homologues (Sarma *et al.*, 2005) however it does contain an arygyl-glycl-aspartic acid (RGD) domain which has been implicated in adhesion. After structural resolution of this protein through x-ray crystallography it was shown that this domain resided in a mobile loop region of the protein at amino acid 140-142 (Zhang *et al.*, 1997).

1.3.4 RGD domain

The RGD domain is crucial for toxin activity. Meinhardt *et al.*, 2002, have shown the importance of this domain for Ptr ToxA by demonstrating that a mutation in the RGD domain resulted in an inability to induce cell death and they concluded that the loss of activity in Ptr ToxA was due to the inability of the mutated RGD domain to bind to a recognition factor. This conclusion was further strengthen by electrolyte leakage

studies that demonstrated that co-infiltration of toxin with an RGD tripeptide, in comparison to toxin alone resulted in a 63% decrease in electrolyte leakage (Meinhardt *et al.*, 2002). Electrolyte leakage is a resulting cause of Ptr ToxA interaction in susceptible plants and is used as a method of early detection of disease (Friesen *et al.*, 2002). However this decrease was only observed within the first couple of hours and after 3.5 hrs the protection was reduced to only 33%, most likely due to the fact that the interacting protein has a higher affinity for Ptr ToxA than the tripeptide.

Ptr Tox A is a single domain protein with two antiparallel beta sheets composed of four strands each that enclose a hydrophobic core (Sarma *et al.*, 2005) and contains a single turn alpha helix between beta 1 and 2. The core of the protein is rather rigid, however the loops are more mobile. Loop B5-B6 protrudes from the structure which is where the RGD domain is located allowing for convenient interactions. The most similar protein structure was identified to be fibronectin II (Sarma *et al.*, 2005).

1.3.5 Homologous Integrin Proteins

Fibronectin is an integrin protein in plants and only since 1998 have integrin proteins been identified in plants (Faik *et al.*, 1998). In mammalian cells, the integrin proteins are also exploited by pathogens which use them as adhesion and binding sites (Isberg and Nhieu, 1994) to a plasma membrane. The RGD domain found in integrin proteins is required for binding to integrin receptors. In mammalian cells, these receptors are involved in signal transduction both into and out of the cell (Coppolino and Dedhar, 2000; Clark and Brugge, 1995) and mediate cell adhesion, migration and invasion (Hynes, 1992). These integrin receptors also mediate cellular processes such as apoptosis by a

complex signaling cascade that involves calcium fluxes and protein phosphorylation and dephosphorylation (Cary *et al.*. 1999; Coppolino and Dedhar, 2000).

1.3.6 Host Interactions

Even though integrin receptors have not been discovered in plants, experiments conducted to determine host contribution to the disease suggest that the same apoptotic signaling cascade activated by integrin receptors is also occurring in plants. Plant signaling inhibitors such as calcium channel blockers, protein kinases and phosphatases resulted in transient protection from toxin action when co-infiltrated with Ptr ToxA. Fifty percent protection was observed with protein kinase inhibitors, staurosporine and K252A (Rasmussen *et al.*, 2004) and inorganic calcium channel blockers ($LaCl_3$ and $CoCl_2$) and protein phosphatase inhibitors such as okadaic acid and calyculina resulted in almost complete protection from Ptr ToxA, within the first 6 hrs of electrolyte leakage measurements.

Thus calcium channels and phosphatase activity plays a crucial role in early toxin action (Rasmussen *et al.*, 2004) suggesting that host signaling is required by the toxin to induce necrotic symptoms. This however is not the only requirement from the host. Once toxin perception occurs, cell death from Ptr ToxA also requires *de novo* gene expression, mRNA, and protein synthesis in the host (Kwon *et al.*, 1998). This implies that signaling cascades are activated and result in the production of proteins, however which proteins and their specific roles are unknown.

1.3.7 Toxin Entry

Ptr ToxA is a protein. This has been exploited in determining its localization in plants and beneficial in aiding in the discovery of Ptr ToxA's mode of entry into plants. Fusion proteins of Ptr ToxA with GFP were made which made it possible to visualize where the toxin was at different points in insensitive and sensitive cultivars (Manning and Ciuffetti, 2005). It was observed using immunoflourescence that toxins entered the cell in sensitive and not the insensitve cultvars. This was further shown when proteases were infiltrated into cultivars treated with Ptr ToxA. After protease treatment, Ptr ToxA was not recovered in the insensitive cultivar but was recovered in the sensitive cultivar. The toxin was unable to cross the cell wall in insensitve cultivars, and was digested by the protease, further suggesting that Ptr ToxA is internalized. It appears that the differentiating factor between insensitive and sensitive lines is internalization suggesting that the Ptr ToxA sensitivity gene is most likely related to protein import.

The toxin resides in the apoplastic space in insensitve cultivars and is prevented from entering the mesophyll cell. In sensitive cultivars the toxin enters the cell and localizes to discreet regions of the cytoplasm and the chloroplast (Manning and Ciuffetti, 2005). Internalization of virulence factors has also been seen with Avr proteins produced by fungi which have been shown to induce cell death intracellularly (Jia *et al.*, 2000; Allen *et al.*, 2004; Dodds *et al.*, 2004). To test if internalization was the only differentiating factor between insensitive and sensitive lines, Ptr ToxA tagged with GUS was coated onto gold beads and shot into cells using biolystics, bypassing the insensitive line's inability to internalize Ptr ToxA. The number of GUS spots present on the leaves shot with Ptr ToxA decreased in comparison to control in both lines indicating that both

sensitive and insensitive cells have the ability to respond similarly to toxin once it is internalized (Manning and Ciufetti, 2005) As well, in the sensitive cultivar Ptr ToxA appears to have a specific destination once it is internalized because immunoflouresence has demonstrated that the toxin will co-localize to chloroplasts.

1.4 Chloroplast

1.4.1 Localization to the Chloroplast

The chloroplast has been speculated to be the site of action for the disease by a number of researchers. Morphological studies have shown a loss in chloroplast integrity and a disruptions of thylakoid structure in Ptr ToxA treated sensitive wheat (Freeman *et al.*, 1995), and a decrease in chloropyll flouresence was seen in toxin treated sensitive leaves (Manning *et al.*, 2004). Furthermore, leaves that were infiltrated with Ptr ToxA do not develop necrosis in regions that are not exposed to light (Manning and Ciufetti, 2005).

Chloroplasts are light harvesting units that use light energy to reduce CO_2 to glucose for plants and are essential for the plant. Light reactions of photosynthesis occur within the thylakoid membrane and dark reactions of photosynthesis occur in the stroma. Chloroplasts are involved in responses to environmental stimuli, abiotic stress and plant pathogen interactions. More specifically, they have been shown to be involved in plant defense responses to bacteria and fungi (Kariola *et al.*, 2005; Yaeno *et al.*, 2004). Often chloroplast involvement in a disease is correlated to an increase in reactive oxygen species and intermediates. The exact mechanism is unknown and a direct interaction has not been demonstrated but when yeast two hybrid studies were conducted (Manning *et*

al., 2007) using Ptr ToxA as bait it interacted with both partial and full length pieces of a chloroplast protein in the Ptr ToxA sensitive wheat leaf library.

1.4.2 Tox A BP1

This binding protein in the chloroplast has been designated Tox A binding protein 1, ToxA BP1 and is present in two forms and in two locations within the organelle. There is a 60-70 kDa complex which is speculated to be a homodimer that resides in the chloroplast membrane and a 45 kDa protein, possibly the proteolytic version of the homodimer in the stroma (Manning *et al.*, 2007). When whole cell extracts were analysed, the 45 kDa protein was found to be the most predominant form but this may be an artifact of the extraction method, thus it is difficult to conclude whether the protein is targeted to the membrane or to the stroma.

Levels of transcript of ToxA BP1 are similar in both sensitive and insensitive cultivars (Manning *et al.*, 2007) and it is important to note that the RGD domin is not required for this interaction. Tox A BP1 and mutated forms of Ptr ToxA in the RGD domain (R140A and D142A) were still capable of interactions (Manning *et al.*, 2007). This provides further evidence that RGD domain is required for protein-protein interactions that allow toxin entry into the mesophyll cells. Therefore, Tox A BP1 is not a determinant of toxin sensitivity but is rather a binding protein that ensures localization of Ptr ToxA to the chloroplast or into the chloroplast: possibly the ultimate target of Ptr ToxA.

1.4.3 Chloroplast and Cell Death

Chloroplasts have been shown to participate in plant programmed cell death involving reactive oxygen species (ROS) (Chen and Dickman, 2004, Gray *et al.*, 2002, Samuilov *et al.*, 2003), however the exact mechanism is unknown. ROS are associated with PCD, and chloroplasts appear to be likely sources of ROS, because the photosynthetic electron transport is accompanied by the reduction of dioxygen to superoxide radicals (Samuilov *et al.*, 2003). They also contain large arrays of ROS scavenging mechanisms (Davletova *et al.* 2005) so they have been implicated in the reversal of cell death as well (van Doorn, 2004). Thus it seems they play various roles in PCD and previous studies have shown that their function in each plant pathogen interaction also varies (van Doorn and Woltering, 2004). The current understanding of plant responses to pathogens is limited but ROS have often been implicated in physiological and biochemical changes of plant response to pathogens (Kariola *et al.*, 2005; Kotchoni and Gachomo, 2006). Thus, it is interesting to note that preliminary studies conducted by V.A. Manning, J.A. Steeves and L.M. Ciuffetti (unpublished data in Manning *et al.*, 2007) indicated that treatments with Ptr ToxA results in the accumulation of reactive oxygen species (ROS) which futhur suggests that chloroplast may be contributing to necrosis through cell death signaling mechanisms such as ROS.

1.4.4 Reactive Oxygen Species

Reactive oxygen species, such as superoxide radicals, hydrogen peroxide and singlet oxygen are naturally occurring by products of normal cellular metabolism

(Kotchoni and Gachomo, 2006). The photoelectron transport chain located in thylakoid membranes of the chloroplast are a major source of ROS (Foyer *et al.*, 1994) although other sources of ROS have also been found and they include cell wall bound peroxidase and mitocondria (Mittler, 2002; Davletova *et al.*, 2005). Reactive oxygen species play important roles in stress inducible genes as well as crucial roles in various different metabolic processes thus it seems likely that various methods of generation of ROS would also be utilized in a plant pathogen interaction depending on the plant and the pathogen interaction. Thus is would be hard to conclude the exact role and effects ROS have in plants infiltrated with Ptr ToxA. Further insight into the complex array of proteins and signalling pathways that are being altered by Ptr ToxA would require proteomics which is a powerful approach for evaluating global protein changes.

1.5 Proteomics

1.5.1 Benefits of Global Evaluation

Changes in gene and protein expression are the key-regulatory mechanisms employed by cells to execute biological functions including responses to biotic and abiotic stresses. In systems where signaling pathways or specific proteins have been suggested to be involved, they can be directly studied, however, when exact mechanisms are unknown, which is often the case, specific evaluation is not an option. In these instances a broad observation of total protein changes is preferred. For this reason, contemporary research has shifted to proteomics as a strategy for quantitfying, evaluating expression and elucidating functions of protein within the whole plant and has quickly

become one of the most powerful tools in deciphering molecular events in an interaction.

A proteome represents the expressed genome however a linear correlation between genes expression and level of protein does not exist (Watson et al. 2003). Therefore analysis of the proteome is beneficial since transcription does not necessarily mean translation and because proteins are subject to post translational modifications that affect regulatory mechanisms through signaling, a change that genomics can not detect. However, proteins involved in signal transduction are often present in a low abundance, so they are not easily detectable (Rossignol *et al.*, 2006). Thus the most idealistic method of investigation would utilize trancriptomics in conjunction with proteomics.

1.5.2 Proteomic tools

Methods of studying the proteome have evolved within the last few years. The traditional and most common method is still based on two dimensional electrophoresis (IEF x SDS-PAGE), which is mainly used in comparative studies. More recently non-2D gel based methods of evaluation such as multidimensional protein identification technology (MudPIT) have been developed, however these approaches are best for organisms with a complete genomic sequence (Rampitsch and Srinivasan, 2006). Proteins of interest in these experiments are identified by mass spectrometry. The best method for evaluating changes within a proteome of a host-pathogen interaction is still 2DE. A proteome is suppose to a represent the "total" protein within a system at a given instant, however due to limitations of two dimensional electrophoresis and extraction methods, only a subset of proteins limited by protein solubility, pI and molecular weights are

resolved. Even without the limitations of 2DE, it is impossible to represent the total protein population because the methods of protein extraction determine the type of proteins detectable for evaluation. The most generic and useful method of protein extraction is TCA-acetone precipitation (Carpentier *et al.*, 2005). Depending on the tissue, profiles may be complex; however protein fractionation and extraction specialized for plant organelles have been used to simplify a protein profile. Reducing protein profile complexity allows for low abundant proteins such as regulatory or modified proteins to be visualized. Restricting analysis to specific organelles is particularly useful for studying plant-pathogen response since organelles such as mitochondria and chloroplasts are often stimulated by pathogen interaction.

1.5.3 Proteomics and Plant-Pathogen Interactions

Studying biotic stress can be problematic since the pathogen's proteome may obscure the proteomes of the host, further complicating the analysis; however it can be done and many discoveries have been made through the use of proteomics. Proteomics has aided the following categorical biotic stress observations: defense and stress related proteins are represented even in the absence of stress (Jamet *et al.*, 2006; Alvarez *et al.*, 2006), the majority of differential proteins present in resistant and susceptible pathotypes fall into two major categories: defense and stress proteins and enzymes involved in carbon and nitrogen metabolism as well as secondary metabolites (Rossignol *et al.*, 2006). Proteomics has also been used as a tool in the characterization and identification of enzymes involved in the synthesis and degradation of key defense related signaling molecules such as salicylic acid, hydrogen peroxide and nitric oxide (Rossignol *et al.*,

2006). In specific studies with elicitor treatment in soya bean cells, oxidative bursts and protein phosphorylation were observed (Slaymaker and Keen, 2004).

Proteomic is a very comprehensive non biased method of evaluating and deciphering molecular events in a plant pathogen interaction, since there are no preconceived notions of what is to be discovered. Proteomic evaluations between plant pathogen interactions are still heavily reliant on 2DE observational comparative changes, which is reasonable since 2D gels allow for simultaneous analysis of a broad range of proteins. Conclusions derived from these studies can then be used as aims for future studies. Thus proteomics is capable of answering questions not readily attainable by any other technology, making it a very exciting area of new discoveries.

1.6 Rationale

Ptr ToxA, the necrosis inducing toxin of *P. tritici-repentis*, is a well characterized host specific toxin. The genetics of toxin sensitivity is well understood and the protein and host interactions have been extensively studied. Signaling inhibitors such as protein kinases, calcium channel blocker and phosphatases were able to retard the initial effects of Ptr ToxA (Rasmussen *et. al.*, 2004), demonstrating that signals are induced. Also, the homology of Ptr ToxA to the RGD motif of integrin protein further suggest the toxin is capable of signalling since intergrins are involved in receptor mediated response (Sarma *et al.*, 2005).

In ToxA- insensitive cultivars Ptr ToxA was incapable of crossing the plasma membrane from the apoplastic space however in ToxA sensitive cultivars Ptr ToxA is internalized and co-localizes to the chloroplast. Chloroplasts are involved in both rapid

cell death and senescence. Therefore, it is possible that chloroplasts may be initiating signals from either pathways to induce necrotic death as a result of interactions with Ptr ToxA. If so, these changes would be observed in the proteome of whole leaves as well as the proteome of isolated chloroplast.

Thus, proteomics was utilized to identify the proteins affected post ToxA interactions leading to cell death, to decipher and formulate molecular events occurring in the plant cell as a result of Ptr ToxA interactions. The elucidation of the molecular events leading to the induction of necrosis would add to the understanding of Ptr ToxA, and aid in the future studies of other proteinacious host specific toxins.

Chapter 2: Materials and Methods

2.1 Growth of Pyrenophora tritici-repentis and Wheat Host Plants

2.1.1 Spore germination

Pyrenophora tritici-repentis (Died.) Drechsler, strain 86-124, Race 2 which produces Ptr ToxA, a necrosis inducing toxin, was obtained from Dr. L. Lamari, Department of Plant Science, University of Manitoba. Dried wheat leaves infected with isolate 86-124 were placed on V8- Potato Dextrose Agar (PDA) consisting of 150 ml of V8 One Shot juice, 10 g of potato dextrose agar (Difco), 3 g $CaCO_3$, 10 g Bacto agar, and 850 ml of distilled water, at 20°C, until mycelia reached a 2cm diameter. At this point mycelia were knocked down with a sterile glass tube and incubated for a 12 h light followed by dark cycle to induce spore production. From this sporulating mass a single spore was picked and plated onto a fresh V8 PDA plate to produce a mycelial lawn 2 cm in diameter, and then the procedure for generating spores was repeated. Spores were collected in sterile water by flooding the plate and gently scraping the surface of the lawn, using a cell culturing scraper. The absorbance of the resulting spore suspension was measured with Klett-Summerson photoelectric colorimeter and the concentration was determined on a premeasured graph of absorbance versus spore concentration of *Pyrenophora tritici-repentis* spore, provided by Dr. A. Tekauz, Agriculture and Agri-Food Canada.

2.1.2 Liquid Culture

Approximately 220,000 spores were added to each of the eight batches of 250 ml solution of Fries Medium consisting of 5 g ammonium tartrate, 1 g NH_4NO_3, 0.5 g

$MgSO_4$, 1.3 g KH_2PO_4, 30 g sucrose, 1.0 g yeast extract, and 2.0 ml trace elements (167 mg, $LiCl_3$, 10 mg $CuCl_2$, 34 mg MoO_4, 72 mg $MnCl_2$, and 80 mg $CoCl_2$ in 1L) (Dhingra and Sinclair, 1985). An alternate culture of a low phosphate Fries media consisting of one tenth the original amount of phosphate was also grown. Approximately 120,000 spores were added to eight batches of 200 ml cultures. Multiple plates of each media were grown in square culture plates (Corning Square bioassay dish, 245 mm x 245 mm) for a total of 3 weeks at 20°C in still culture. At 2 weeks, 1 ml of the culture was aspirated, diluted 1:1 and infiltrated into the first and second leaf of wheat seedlings using a Hagborg device (Hagborg, 1970). Leaves were examined one and two days post-infiltration for necrosis. Cultures which tested positive for necrosis within this time period were incubated for a total of three weeks, the others were discarded.

2.1.3 Plant growth conditions

Toxin sensitive wheat (*Triticum aestivum L.)* cultivar, Glenlea and toxin insensitive wheat cultivar, Amazon, were grown from seed in greenhouses with 16hr of light at 21°C and 8 hr of dark at 16°C. Plants were grown for two weeks for bioassays and three weeks for chloroplast extractions.

2.1.4 Plant infiltration

Purified toxin, 40ng/μl in 10mM sodium acetate pH 5.0 was diluted and infiltrated into wheat leaves with a Hagborg device (Hagborg, 1970) which floods the apoplastic space of the leaves. Approximately 100 μl of solution was injected into a 3 cm leaf region. The infiltrated region was marked with India ink.

2.2 Ptr ToxA Purification

2.2.1 Anion exchange chromatography

Liquid from the toxin-producing cultures was collected after 3 weeks as follows: Media was filtered through two layers of Whatmann 1 paper followed by a 0.45 μm filter (Millipore, nylon membrane). The filtrate was then concentrated by centrifugation through 5000 molecular weight cut off (MWCO) spin columns (Vivaspin) to one twentieth of their original volume and diluted 1 in10 with 10 mM ammonium acetate pH 5.0 to lower the conductivity. The diluted 150 ml solution was then loaded onto a 5 ml carboxymethyl (CM) cellulose column (Amersham) attached to an Akta Prime chromatography unit. The column was run with 10 mM sodium acetate pH 4.0 at 5ml/min and the eluant was eluted off with a 100 ml linear gradient of 0- 300mM NaCl in 10 mM sodium acetate pH 5.0. The eluant was monitored at 280nm.

The highest absorbing fractions from each peak were diluted 1:50 with double distilled water and 100 μl was infiltrated into sensitive (Glenlea) and insensitive (Amazon) wheat cultivars; in addition, 50 μl of each fraction was collected and separated by electrophoresis on a 10 cm Tris-tricine gel to estimate the population and purity of soluble protein in each fraction. Once the active fraction was identified, the fractions surrounding it were also analyzed by Tris-tricine gel electrophoresis and those fractions that contained a greater concentration of toxin relative to other proteins present in the solution were pooled, fraction 23-30, and desalted using 5000 MWCO filters (Vivaspin) with three buffer changes of 10mM sodium acetate pH 5.0. This desalted solution was reloaded for a second cycle of CM cellulose chromatography to further purify the toxin. Purified active fractions were collected from both the Fries media and the low phosphate

media. These were analyzed on a 10-20% Tris-tricine gradient gel to assess purity and relative molecular weight of the isolated protein.

2.2.2 Desalting

Active fractions from the low phosphate Fries media were pooled and desalted on Sephadex G-25 (Amersham HiPrep 26/10) attached to Biologic HR (BioRad) workstation, using 10 mM sodium acetate pH 5.0. The fractions from the eluted peak were pooled in two sets, a high protein set and a low protein set. A dilution series of 1:10, 1:50, 1:100, 1:200 and 1:500 was made with deionized water and was infiltrated into wheat cultivar Glenlea to assess the activity.

2.3 Toxin Concentration Calculation

The absorbance of the solution was measured at a wavelength of 280nm and the protein concentration was calculated using the equation A=Elc with an E_M=20,800 for the 13.9kDa Ptr ToxA protein (Ballance *et. al.*, 1989).

2.4 Tris Tricine Electrophoresis

2.4.1 Tris-Tricine gels

Gels were made by pouring a separating gel solution (1 M Tris, 0.1% (w/v) sodium dodecyl sulfate, 12% acrylamide, 0.0009% (v/v) TEMED, and 0.09% (w/v) ammonium persulfate) between two mini plates overlayed with water saturated n-butanol. Once polymerized, the n-butanol was rinsed off, and a stacking gel was poured above the base gel with a well comb placed in prior to polymerization. Once polymerized, gels were

snapped into the mini protean III (BioRad) plate holder, which form the upper chamber of this unit. Upper cathode buffer (0.1 M Tris, 0.1 M Tricine, 0.1% (w/v) SDS) was poured into the chamber and samples were loaded into the buffer-filled wells. Anode buffer (0.02 M Tris pH 8.9) was poured into the outer chamber.

2.4.2 Sample preparation

The 30 μl of sample consisting of 20 μl of each peak fraction was mixed with 10μl of 3 X SDS sample buffer (0.16 M Tris-HCl pH 6.8, 12% (w/v) SDS, 30% (v/v) glycerol, and 0.096% (v/v) serva blue) boiled for 5 mins and loaded onto the gel. An aliquot of 3μl of low molecular weight standards (Page ruler protein ladder, Fermentas) was also loaded along with the samples.

2.5 Leaf Protein Extraction

Infiltrated regions of whole leaves were collected one to four days post infiltration and immediately frozen in liquid nitrogen after each collection. Mortars and pestles were pre-cooled with liquid nitrogen prior to grinding. Three infiltrated frozen leaf regions (approx. 1g) were ground into a fine powder until the liquid nitrogen evaporated. This process was repeated twice and the ground tissue was transferred into a 15 ml corex tube. Protein was precipitated from the fine leaf powder by adding 10 ml of acetone chilled to -20°C, with 10% (w/v) TCA and 0.07% (w/v) DTT and inverting three times or until precipitants appeared. This was then left overnight at -20°C to ensure maximum precipitation. Samples were centrifuged at 10,000g for 20 min at 4°C and the pellets were washed in 10 ml of acetone with 0.07% (w/v) DTT. The wash was then

repeated seven times. At the final wash the pH of the supernatant was determined after the removal of acetone to ensure that all traces of TCA had been eliminated. Once the pH of the supernatant was equivalent to that of water, the pellet was dried under a stream of nitrogen gas and stored at -80°C.

2.6 Two Dimensional Gel Eletrophoresis

2.6.1 Protein Preparation

Dried proteins from the -80°C freezer were left to equilibriate to room temperature, and then dissolved in 2ml of isoelectrofocusing (IEF) buffer (7 M urea, 2 M thiourea, 20 mM DTT, 4% (w/v) CHAPS, 0.02% (v/v), 3-10 40% stock ampholyte (Biolyte 3-10, BioRad). Proteins were shaken continuously for 2 hrs followed by sonication with a microtip sonicator, (Misonix) for a total of 25s, with 5s pauses between each 5s interval; during this time the sample was kept in an ice slurry. After sonication the remaining solution was centrifuged at 10,000g for 20 min and the protein solution was added to 2 ml 5000MWC spin columns (Vivaspin) to reduce the volume to 1/10 the original volume. Then fresh buffer was added to bring it back to the original volume. This was repeated twice for a total of three buffer exchanges to remove any residual salts in the sample. During the final exchange, the solution was concentrated to approximately 400 μl and this concentrate was centrifuged at 80,000g for 30 min on an ultracentrifuge (Airfuge, Beckman Coulter). Supernatant was removed and the protein concentration was determined by Bradford protein assay (Bradford, 1976).

2.6.2 Isoelectric Focusing

The volume required to contain 500 μg of protein was calculated and suitable aliquots were dispensed into silconized tubes and made up to a volume of 450 μl with IEF buffer stained with bromophenol blue. This was placed evenly across a well of a custom built Teflon IEF rehydration tray. IEF strips pH 4-7, 24 cm (Amersham) were then placed gently on top of the solution and overlayed with mineral oil to prevent evaporation. The strips were left overnight at 20°C to rehydrate. The rehydrated strips were rinsed briefly with double distilled water then placed on pre-moistened Whatmann paper and layered with another three sheets of moist Whatmann paper and finally with two dry Whatmann sheets to remove excess water. The strips were then placed in wells of the isoelectrofocusing unit (Amersham Multiphor II) and focused for a total of 58.9 kVh at 20°C with the following program; 250 V for 4 hr, 250 V for 0.5 hr, 1200 V for 4 hr, 1200 V for 0.5 hr, 3500 V for 4.5 hr , and 3500 V for 12.5 hr.

2.6.3 Strip preparation for Two Dimensional Gel Electrophoresis

Resolved strips were reduced in two changes of equilibriation buffer (50 mM Tris-HCl pH 8.8, 5.9 M Urea, 0.3%(v/v) glycerol, 2%(w/v)SDS) with 1% (w/v) DTT for 8 min followed by another two washes for 8 min in equilibration buffer (50 mM Tris-HCl pH 8.8, 5.9 M Urea, 0.3%(v/v) glycerol, 2%(w/v) SDS) with 2.5% (w/v) iodoacetamide. The strips were then rinsed with electrode buffer (25 mM Tris, 192 mM glycine, and 0.1% (w/v) SDS) and placed on the second dimension gels.

2.6.4 Electrophoresis

The second dimension was carried out in an Ettan Dalt 6 unit (Amersham) according to the manufacturers instruction with some modifications. Each glass plate was rinsed in water, then polished with 95% ethanol and placed in the pouring apparatus. A 12% acrylamide gel solution, 0.375 M Tris- HCl pH 8.8, and 1% (w/v) ammonium persulfate was degassed and chilled on ice while 0.1% (w/v) SDS and 0.00013% (v/v) TEMED was added and mixed into the solution. After gentle mixing, the solution was poured into the gel apparatus leaving 1cm of space between the gel and the top of the plate. The gels were then overlayed with water-saturated n-butanol and left to polymerize for a minimum of 90 min. Once the gels had set, the water-saturated n-butanol was decanted and the gels were rinsed with distilled water. The interface was left to dry for 1 min before positioning the strip tightly at the interface of the gel. The strip was held in place by solidified low melt agarose (1% (w/v) low melt agarose, 25 mM Tris, 192 mM glycine, and 0.1% (v/v) SDS). The electrophoresis unit was then assembled (Ettan Dalt 6 eletrophoresis unit, Amersham) and electrode buffer (25 mM Tris, 192 mM glycine, and 0.1% (w/v) SDS) was added to the upper and bottom buffer chamber and kept at a constant temperature of 22.5 °C. Gels were electrophoresed at 2 W for 1.5 hr to allow for slow entry of proteins into the gel, followed by 17 W per gel until the dye front had traveled the distance of the gel.

2.6.5 Coomassie Blue Staining

Gels were fixed in 12.5% (w/v) trichloroacetic acid for 1hr and then stained with 5% (w/v) R-250 Commassie in 95% ethanol, to a final concentration of 0.07%(w/v) and

left overnight with constant shaking. The dye was removed and the background destained with double distilled water for 8 h with gentle agitation. Once destained, gels were visually compared for spot differences.

2.7 Chloroplast Experimentation

2.7.1 Chloroplast isolation

Three week old wheat leaves were harvested and cut into 1cm^2 pieces and mixed with ice-cold homogenization buffer (0.33 M Sorbitol, 50 mM Tris pH 7.5, 0.4 mM KCl, 0.04 mM Na_2EDTA, 0.1% (w/v) BSA, 1% (w/v) PVP, 5 mM Na-isoascorbate). A homogenizer (Polytron) was used to blend the leaves with four 5s pulses. The homogenate was filtered through four layers of mira cloth to remove leaf debris and filtered again through another eight layers of cheese cloth. Chloroplasts were then pelleted at 1000g for 1min in 30ml glass corex tubes in an SS-34 rotor (Sorvall). The supernatant was decanted and the pellet was washed in resuspension media (0.33 M Sorbitol, 2 mM Na_2EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 50 mM HEPES pH 7.6) and centrifuged at 1000 g for 1 min.

After the supernatant was removed, the chloroplasts were resuspended in 2ml of the resuspension media and transferred into a 50 ml Corning tube. This solution was underlayed with 10mls of 40% (v/v) Percoll in resuspension buffer followed by 5ml of 80% (v/v) Percoll in resuspension buffer and the resulting gradient was centrifuged at 2,500g for 15 min in a SH-3000 rotor (Sorvall) to separate the intact and broken chloroplasts. Intact chloroplasts formed a band at the 40%/80% interface of the Percoll gradient and were aspirated into another 50 ml Corning tube. Residual Percoll was

removed from the suspension by washing with 10 volumes of resuspension media, followed by centrifugation at 1,500g for 3 min. The chloroplast pellet was resuspended in 2ml of chloroplast media (50 mM Hepes-KOH pH 7.6 at 4 °C, 0.33 M Sorbitol, 2 mM Na_2EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM $NaNO_3$, 1 mM NaH_2PO_4, 2 mM Na-isoascorbate) and kept on ice until use.

2.7.2 Chloroplast inoculation with purified Ptr ToxA

The amount of toxin added to each chloroplast suspension was determined by an approximate calculation. Wheat leaves contain 124,000 mesophyll cells/cm^2 (Jellings and Leech, 1982) and since chloroplast numbers vary in wheat mesophyll cells depending on the cultivar (Pyke and Leech, 1987), an average was taken from 15 cultivars, and this number was determined to be 143.6 chloroplast/mesophyll cell. The average length of inoculated leaves was 3.5 cm with a width 0.5 cm, thus contained an area of 1.75 cm^2. Based on these values there are approximately 31 million chloroplasts in the infected region. For the leaf infiltration approximately 100 μl was infiltrated into each 3 cm leaf region at a concentration of 40 ng/μl, thus the ratio of chloroplast to toxin was determined to be approximately 390,000 chloroplast/ng of toxin. The concentration of the chloroplast suspension was determined by counting chloroplasts using a hemocytometer. The chloroplast suspensions were brought to room temperature and treated with 1 ng of toxin for every 390,000 chloroplasts in the suspension, the same volume of buffer (10 mM sodium acetate pH 5.0) was added to the control sample and incubated for 1 h with continuous shaking under bright lighting. After the incubation proteins were precipitated from the solution with 10 ml of acetone, 10% (w/v) TCA, and

0.07% (w/v) DTT. These precipitated protein samples were processed the same way as the leaf samples.

2.8 Mass Spectrometry

2.8.1 Protein spot processing for linear ion trap Mass Spectrometry

Protein spots that showed upregulation or downregulation were cut out of gels with a scalpel and washed in 50% methanol. Identical spots from each gel were pooled and spots larger than 1mm^2 were cut down to this size, washed in 200 μl of water and shaken for 10min. The gel pieces were centrifuged and the supernatant was discarded. The gels were then washed with 100 μl of 100 mM NH_4HCO_3, with continual mixing with a vortex for 10 min; after which one volume of acetonitrile was added and then mixed for another 10 min. The supernatant was removed and gel pieces were dried in a speed vacuum for exactly 5 min. Gel pieces were then reduced in 100 μl of 100 mM NH_4HCO_3, 10 mM DTT for 45mins at 56°C, and alkylated in 100 μl 100 mM NH_4HCO_3, 55 mM iodoacetamide for 30 min in the dark with occasional mixing. The solution was decanted and gel pieces were washed with 100 mM NH_4HCO_3 and vortexed for another 10min to which one volume of acetonitrile was added. It was then washed twice in 200 μl of 50% (v/v) acteonitrile and 50 mM NH_4HCO_3. After the final wash the gels were dried for 15 min in the speed vacuum. Gel pieces were rehydrated in a 100 mM NH_4HCO_3, 10% (v/v) acetonitrile, 2.5 mM $CaCl_2$ with 12ng/μl trypsin for 30 min on ice. After 15 min spots were checked to ensure that a sufficient amount of solution had been added. Tubes were then sealed with parafilm and digested overnight at 37°C.

Peptides were then extracted from the gels with a series of solutions with 10min of vortex mixing between each solution, starting with 100 μl of 5% (v/v) formic acid, followed by 100 μl of 1% (v/v) formic acid in 5% (v/v) acetonitrile, then 50 μl 1% (v/v) formic acid in 60% (v/v) acetonitrile, and finally with 100 μl of 1% (v/v) formic acid in 99% (v/v) acetonitrile. Each solution was collected in a new siliconized tube and each was dried in speed vacuum.

2.8.2 Mass Spectrometry Analysis and Protein Identification

Mass Spectrometry experimentation was conducted by Tao Fan at the Cereal Research Center (Agriculture and Agri-Food Canada) on a LTQ linear ion trap mass spectrometer. Briefly: Tryptic peptides samples from each spot were applied with an online C_{18} column and analyzed using a LTQ linear ion trap mass spectrophotometer (Thermo Finnigan). Eluants were directly introduced into the LTQ mass spectrometer via electrospray ionization. Each full mass spectrometry (MS) scan was followed by five tandem mass spectrometry (MS/MS) scans of the most intense ion peaks, with dynamic exclusion to increase coverage.

Protein identification of the MS-MS spectra was preformed by MASCOT search engine (Matrix Science, London, UK). The following parameters were set: a monoisotopic mass accuracy of $\pm$ 2 Da; up to one missed cleavage; peptide charge of +1, +2, and +3, a fixed modification of carbamidomethyl (cys) and variable modifications included oxidation of methionine, deamidation of glutamine and asparagines. Once these parameters were set tandem mass spectra were queried against *Viridiplantae* of the non redundant NCBI database.

Chapter 3: Results

3.1 Isolating *Pyrenophora tritici-repentis* ToxA

Pyrenophora tritici-repentis toxA was isolated from low phosphate Fries media. Two types of media were used to isolate the toxin in liquid culture, Fries media and Fries media with one tenth the phosphate, however only the low phosphate media successfully induced toxin release into the media. The diluted concentrate of the liquid culture from the two types of media produced two different elution profiles when separated by ion exchange chromatography on a CM cellulose column (Figure 1) as well as two different protein populations in the peak fractions, when resolved on Tris-tricine gel (Figure 2) Bioassays of the peak fractions were checked 5 days after infiltration for necrotic lesions (Figure 3).

Reactive fractions from the low phosphate media showed only signs of necrosis while the reactive fractions of the Fries media showed signs of both chlorosis and necrosis. The corresponding peak to Fraction 26 displayed the greatest amount of necrotic death reaching complete desiccation in the infiltrated region within 5 days (Figure 3A), while the two shoulders surrounding peak 26 displayed a lesser degree of necrotic death. This variation of necrosis appears to be correlated to the amount of protein (marked with arrow in Figure 2) that existed in each fraction.

Such a clear correlation between necrosis and a protein band of the fractions was not seen with the peak fractions of Fries Media. Although necrosis was observed in fraction 24 (Figure 3B) of the infiltrated region it also resulted in yellowing of the leaf above the infiltrated region, an unexpected observation since sensitivity to Ptr ToxA is

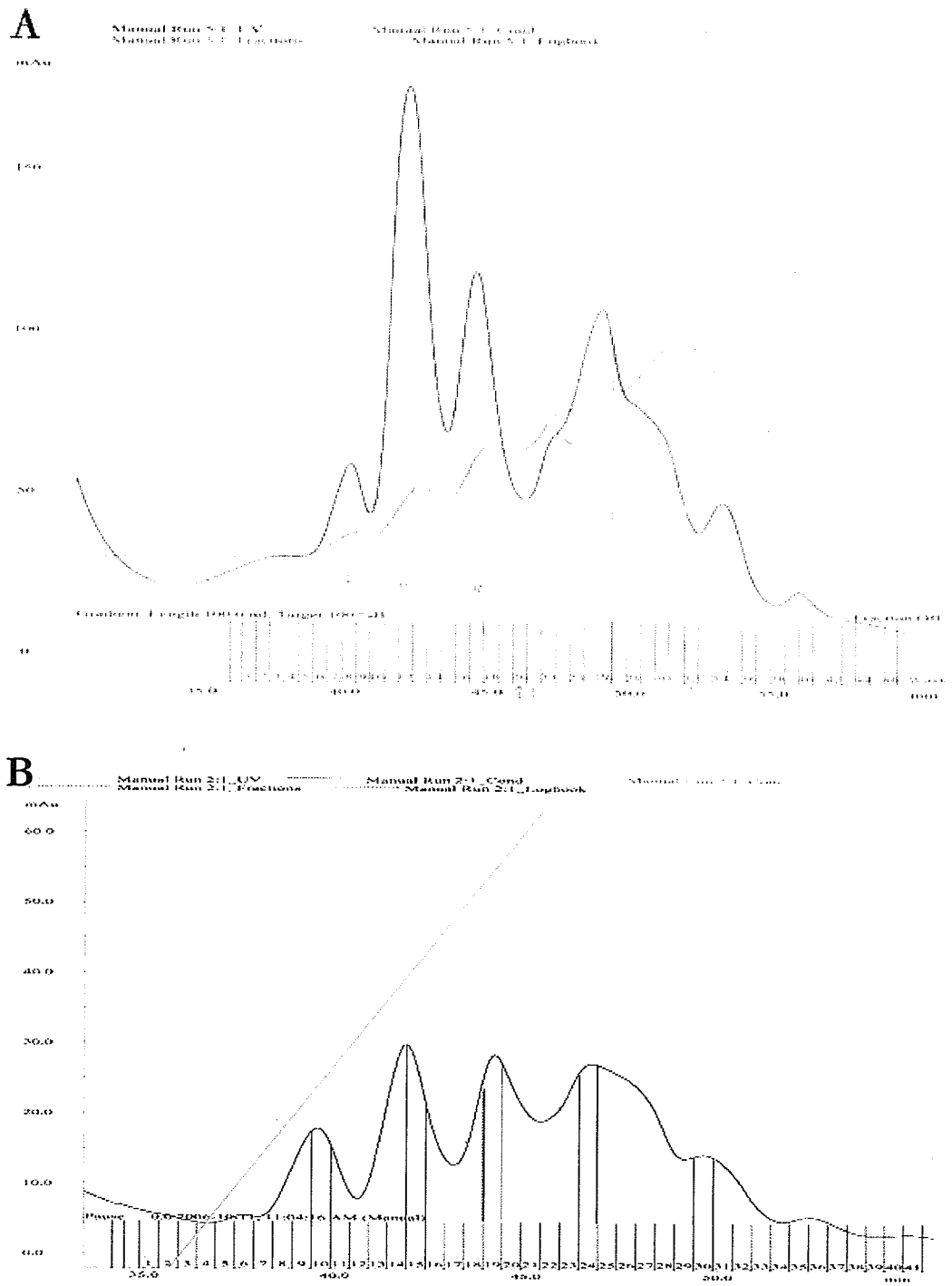
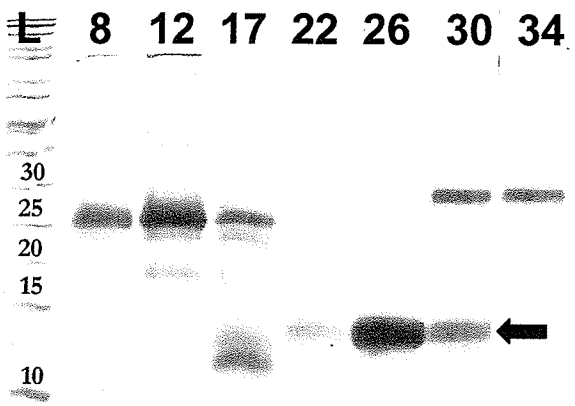


Figure 1: Chromatograms of low phosphate and regular Fries media fractions from CM Celluose column. Concentrated fungal culture in A) low phosphate fries media and B) regular phosphate media were eluted by ion exchange chromatography from CM cellulose column using a linear gradient from 0- 300mM NaCl in10mM sodium acetate, represented by the green line. Elutions were monitored at 280nm. Numbers in red are fraction numbers.

A)



B)

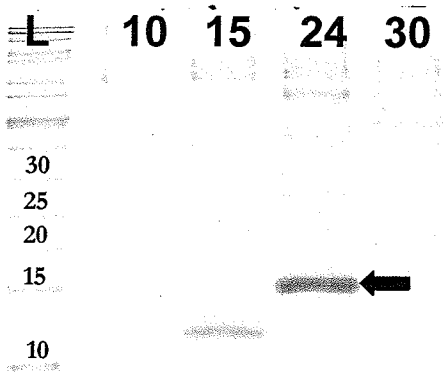


Figure 2: SDS-PAGE of soluble proteins in selected peak fractions of low phosphate and regular Fries media culture eluted from CM celluose column. Selected fractions of A) low phosphate Fries media and B) regular phosphate media fungal cultures eluted from CM cellulose column were separated on 12.5% Tris-tricine gels and stained with Coomassie brilliant blue. L= ladder and numbers are representative of the fractions. The molecular weights are given in kDa. ⬅ Represents band of interest

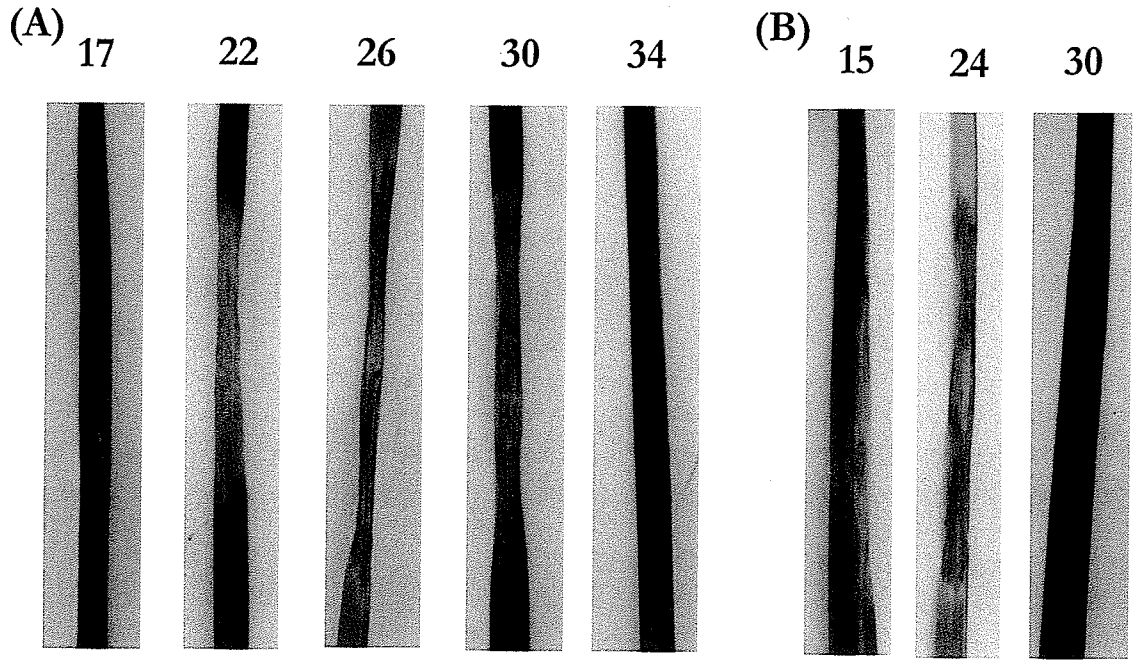


Figure 3: Bioassay of selected peak fractions. Resulting necrotic lesions after infiltration of 1:100 dilution of selected peak fractions after (A) 5 days with low phosphate Fries media culture and (B) 8 days with regular phosphate media culture.

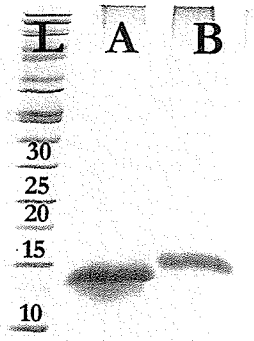


Figure 4: Comparison of purified protein from low phosphate Fries media and regular Fries media on SDS-PAGE. Purified proteins from active fractions of (A) low phosphate Fries media and (B) regular Fries media were resolved on a 10-20% Tris-tricine gel and stained with Coomassie brilliant blue. L= Ladder, the molecular weights are given in KDa.

tissue specific for mesophyll cells and not the vascular tissue (Ballance and Lamari, 1998), and yellowing above the infiltrated region suggests that the vascular tissue may have also been damaged. A slight reaction is also seen in fraction 15 however the reaction appears to be more chlorotic than necrotic.

The most abundant protein band in both media (Figure 2) had a relative molecular weight just under the 15 kDa band of the ladder (low molecular weight pageruler, Fermentas), the expected region for purified Ptr ToxA to reside. However the two bands in Fries media had two different molecular weights, thus further analysis was required to determine which media contained the toxin of interest or if both proteins were Ptr ToxA but different forms.

The pooled fractions containing active Ptr ToxA of each media were compared on a Tris-tricine gradient gel (Figure 4). Based on the relative molecular weight of each protein it was evident that the two isolated proteins were different. This was further verified by *de novo* sequencing (Table 1) which conclusively proved that two proteins with different sequences were isolated. Ptr ToxA was isolated in the low phosphate media, as expected based on the observations of the bioassay and it is interesting to note that the protein isolated from Fries media has unique sequence that failed to yield any credible matches in the NCBI database.

3.2 Proteomic Changes in Wheat Leaves

Necrosis was observed in Glenlea leaves infiltrated with Ptr ToxA and the lesion progressively increased from day one to day three (Figure 5) symptoms were not manifested in either cultivars infiltrated with control buffer, 10mM sodium acetate pH

Table 1: Proteins identified by mass spectrometry **(Ptr ToxA)**

Matrix-assisted laser desorption/ionization–time of flight tandem mass spectrometry (MALDI-MS/MS)

Identitify	**Media type**	**DB #**	**Taxonomy**	**Sequence Tags (from MS/MS spectra)**	**Parent ion, m/z**
Ptr Necrosis Toxin	Low phosphate	GI:1794294	*Pyrenophora tritici-repentis*	LIITQWDNTVTR (Mascot) GDVYELFGDYALIQGR (Mascot) GSFCLNIR (Mascot)	1459.755 1815.844 966.442
Unknown	Regular	Unknown sequence identity (NCBI May 2007)		FEQEYVQDTTTFVTGSSYMDR (de novo) TLQVGQPNVAGLPASCK (de novo) VMHLMAPKHNLASGGFR (de novo) QNPVADNNLQQEDYNDLR (de novo, part)	2504.093 2199.940 2456.075 3104.261

*Table of MALDI-TOF mass spectrometry analysis provided by Dr. N. Bykova, Cereal Research Center, Agriculture and Agri-Foods Canada

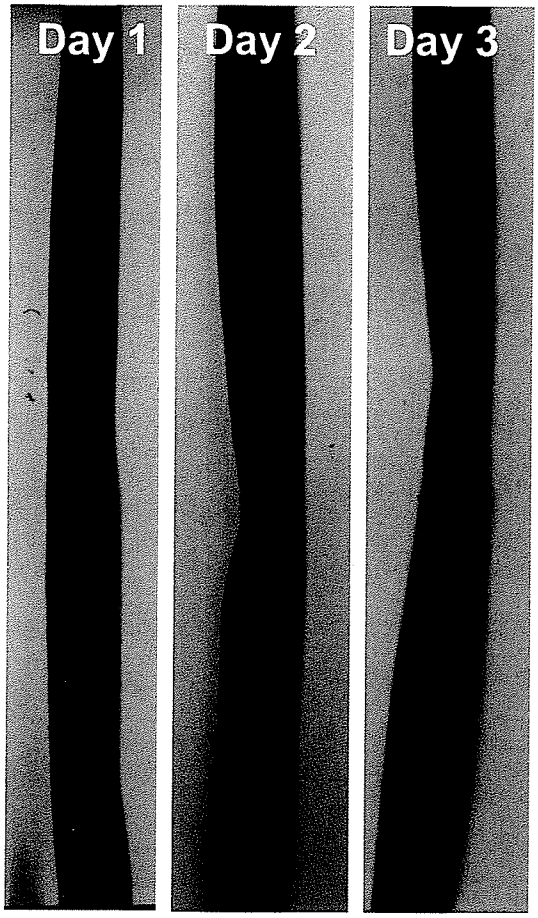


Figure 5: Necrosis progression one to three days post infiltration. Wheat leaves were infiltrated with 1:50 dilution of a 40ng/μl solution of Ptr ToxA and collected at one to three days post infiltration

4.0, or toxin insensitive cultivars infiltrated with Ptr ToxA. Proteins extracted from wheat leaves of each time point were focused by their isoelectric charge on a pH 4-7 gradient strip and separated by molecular weight by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 6 -11, Glenlea 2DE and Figure 12-17, Amazon 2DE). These 2DE gels were used to identify differences in the progression of protein expression from day 1 to day 3 between cultivar treated with toxin and control buffer, 10 mM sodium acetate pH 5.0. However proteome coverage is variable because it is dependant on the sample and the soluble protein extracted from them, thus two independent experiments, starting from the leaf infiltration stage to the 2DE analysis, were conducted for both Amazon and Glenlea; only protein changes observed in both replicates were considered and analyzed.

Up to 70 changes in spot intensity were observed between toxin treated Glenlea and control buffer treated Glenlea, within one of the replicates, however only 20 spots out of the 70 were reproducible. Since protein changes were visually inspected and there were subtle variations in protein loading, only spots that demonstrated visually indisputable changes with similar trends throughout the time points were selected (Figure 18). As a result only nine protein spots were analyzed out of the 20 selected. The same comparison was done with Amazon, the insensitive cultivar, even though fewer changes were initially observed. Nine proteins spots were chosen for analysis (Figure 19).

Peptides extracted from the trypsin digested spots were analyzed by MS/MS after separation on a nano-HPLC and identified by Mascot. Each identified protein had several peptide matches ranging from 10 - 77% coverage (Table 2, Glenlea & Table 3, Amazon). The proteins found in Glenlea and Amazon, were identified in *Triticum*

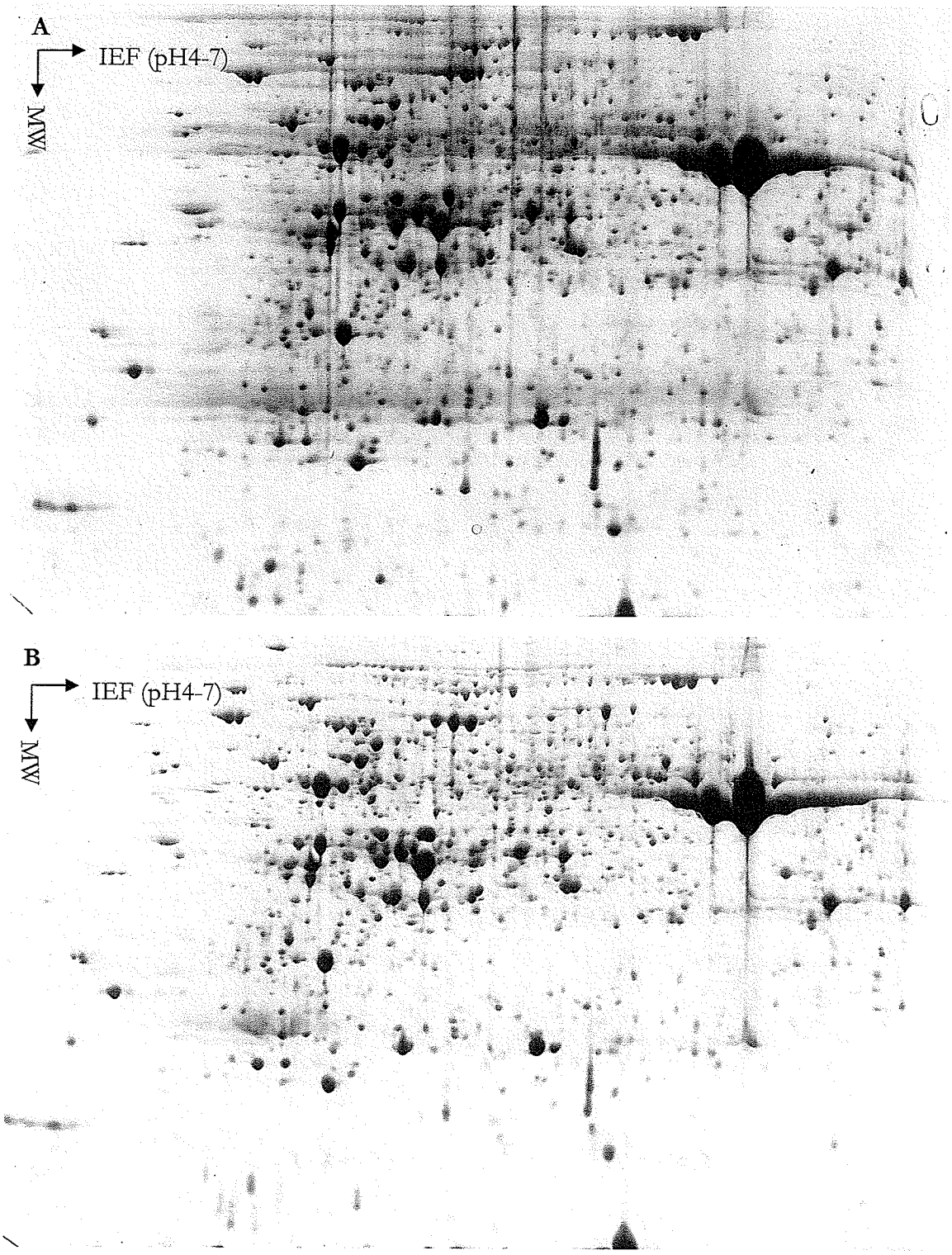


Figure 6. 2-DE separation of total soluble protein from Glenlea wheat leaves 1 day post infiltration with control buffer. Protein from extracted Glenlea wheat leaves infiltrated with 1:50 dilution of 10mM sodium acetate pH 4.0, 1 day post infiltration were resolved on pH 4-7 strips in the first dimension and separated on 12% SDS PAGE in the second dimension, and stained with Coomassie brilliant blue. (A) biological control replicate 1 and (B) biological control replicate 2.

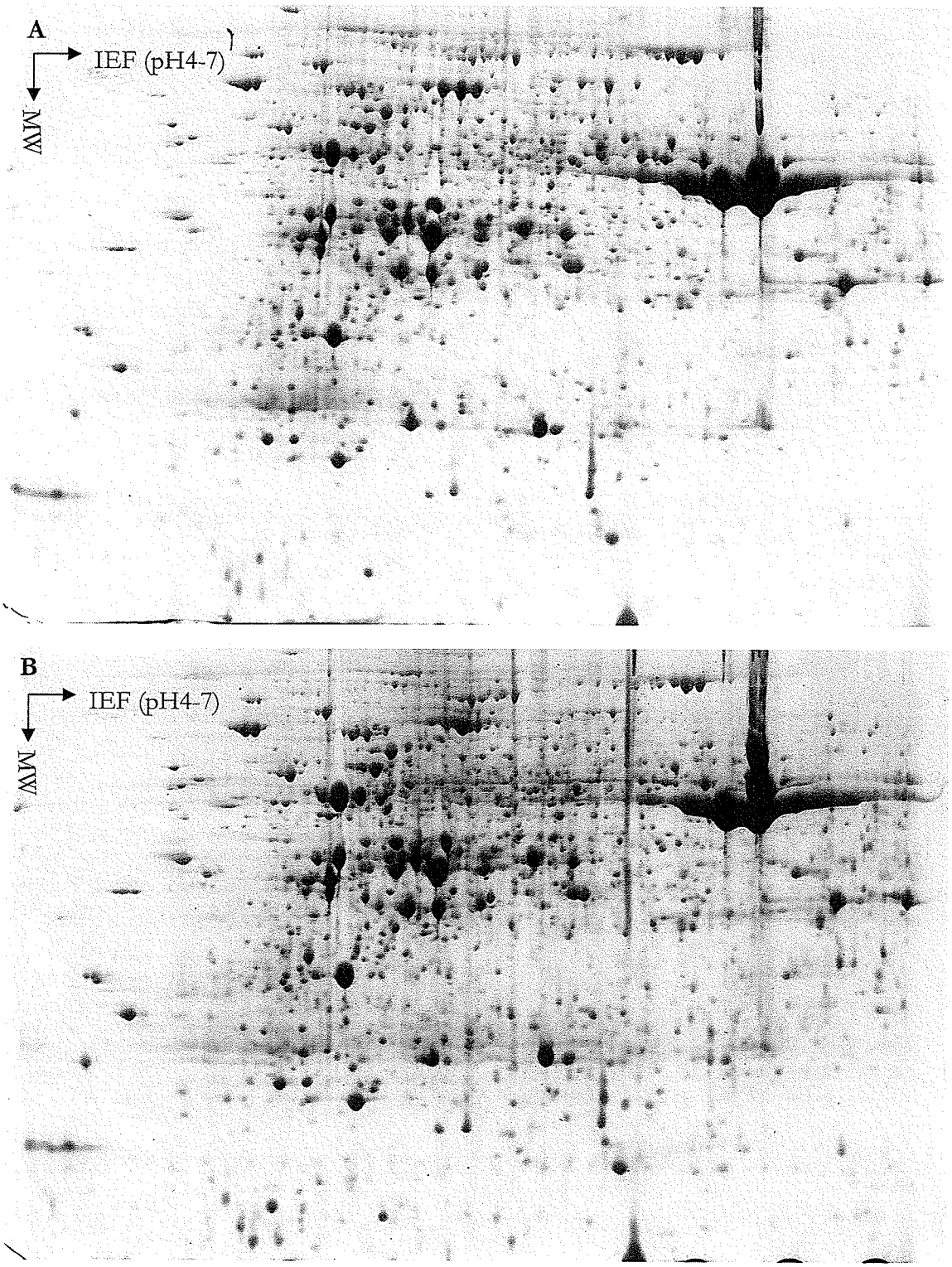


Figure 7. 2-DE separation of total soluble protein from Glenlea wheat leaves 2 days post infiltration with control buffer. Protein from extracted Glenlea wheat leaves infiltrated with 1:50 dilution of 10mM sodium acetate pH 4.0, 2 days post infiltration were resolved on pH 4-7 strips in the first dimension and separated on 12% SDS PAGE in the second dimension, and stained with Coomassie brilliant blue. (A) biological control replicate 1 and (B) biological control replicate 2.

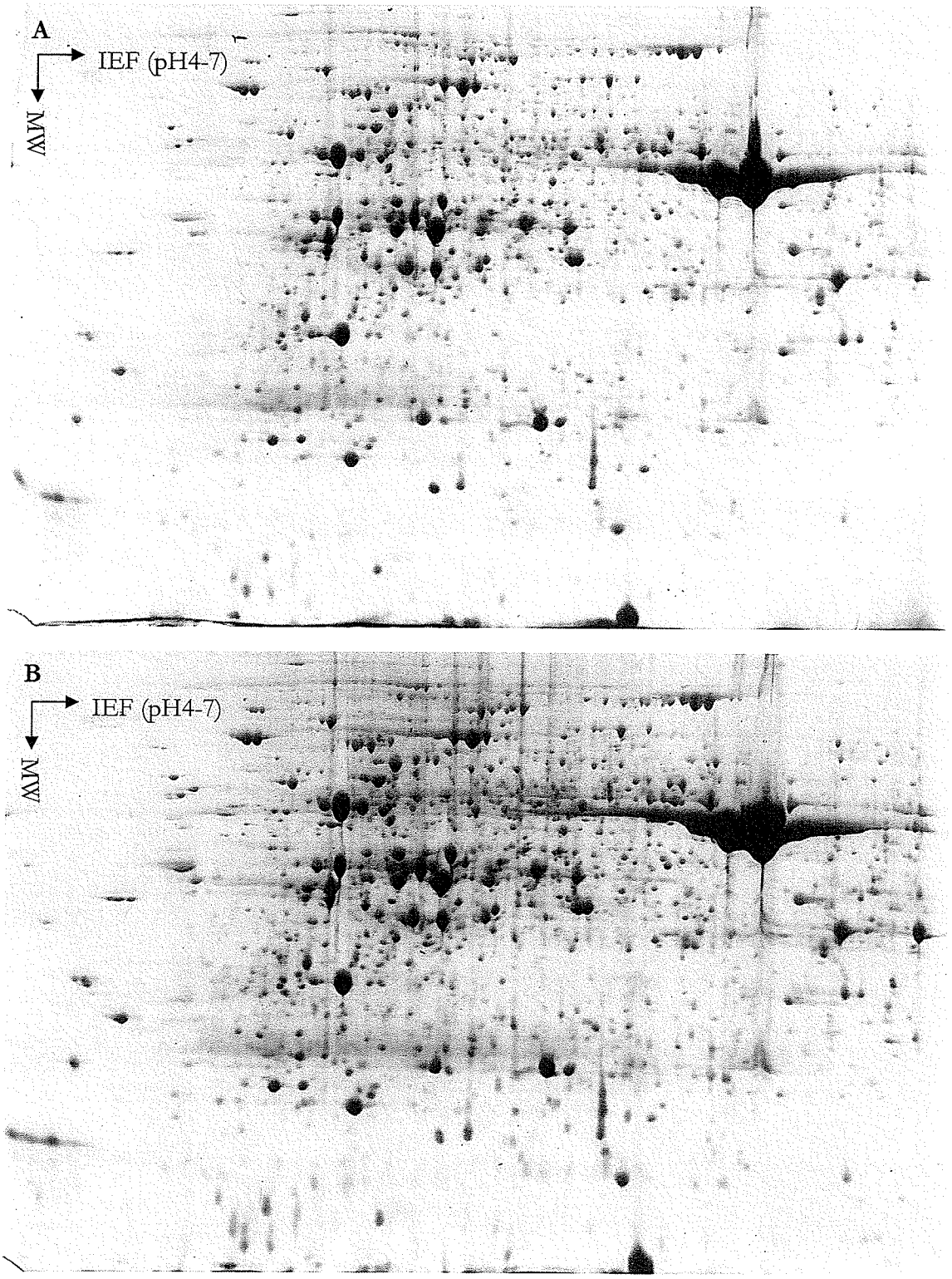


Figure 8. 2-DE separation of total soluble protein from Glenlea wheat leaves 3 days post infiltration with control buffer. Protein from extracted Glenlea wheat leaves infiltrated with 1:50 dilution of 10mM sodium acetate pH 4.0, 3 days post infiltration were resolved on pH 4-7 strips in the first dimension and separated on 12% SDS PAGE in the second dimension, and stained with Coomassie brilliant blue. (A) biological control replicate 1 and (B) biological control replicate 2.

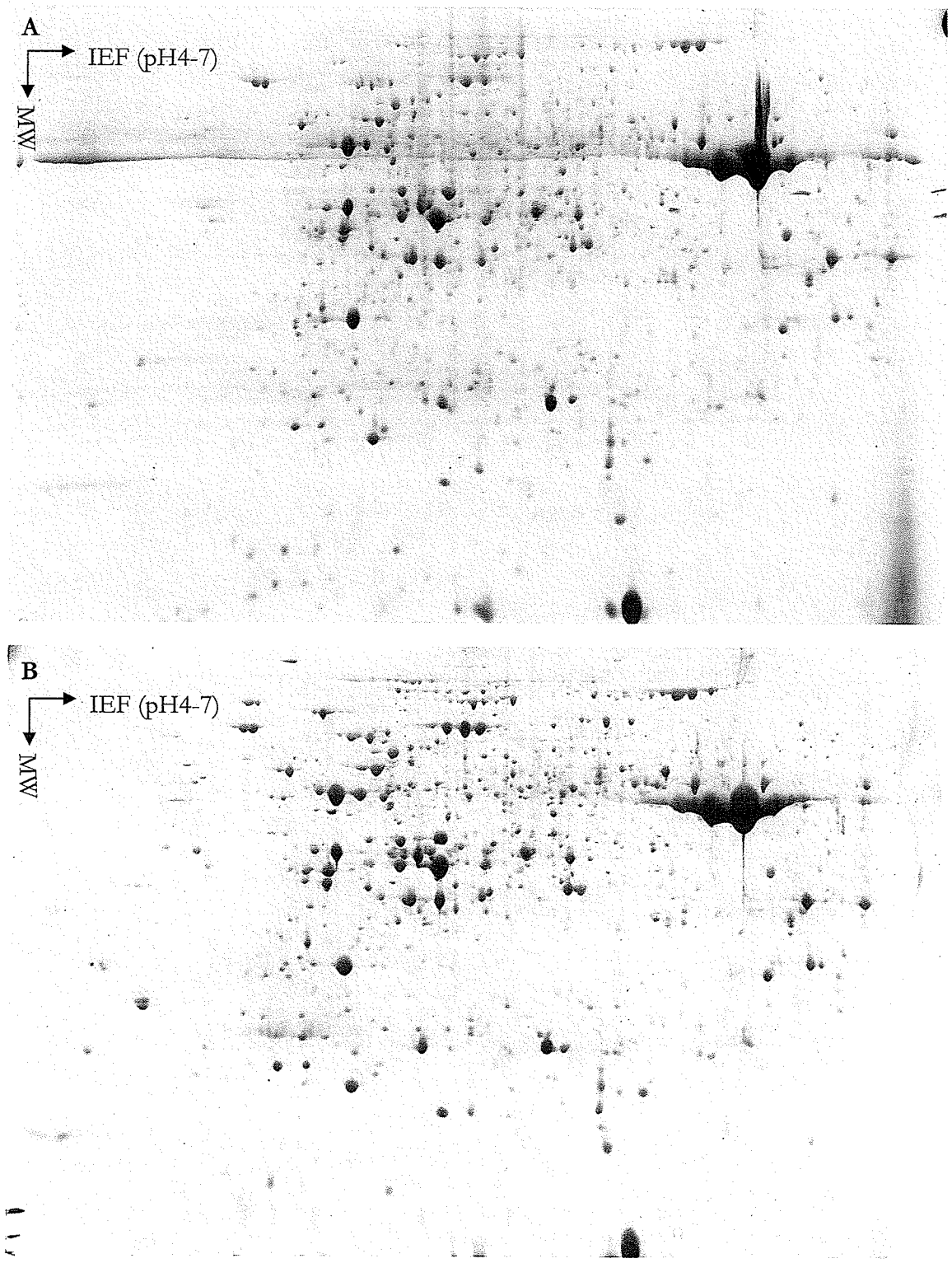


Figure 9. 2-DE separation of total soluble protein from Glenlea wheat leaves 1 day post infiltration with Ptr ToxA. Protein from extracted Glenlea wheat leaves infiltrated with a 1:50 dilution of 40μg/μl of Ptr ToxA, 1 day post infiltration were resolved on pH 4-7 strips in the first dimension and separated on 12% SDS PAGE in the second dimension, and stained with Coomassie brilliant blue. (A) biological control replicate 1 and (B) biological control replicate 2.

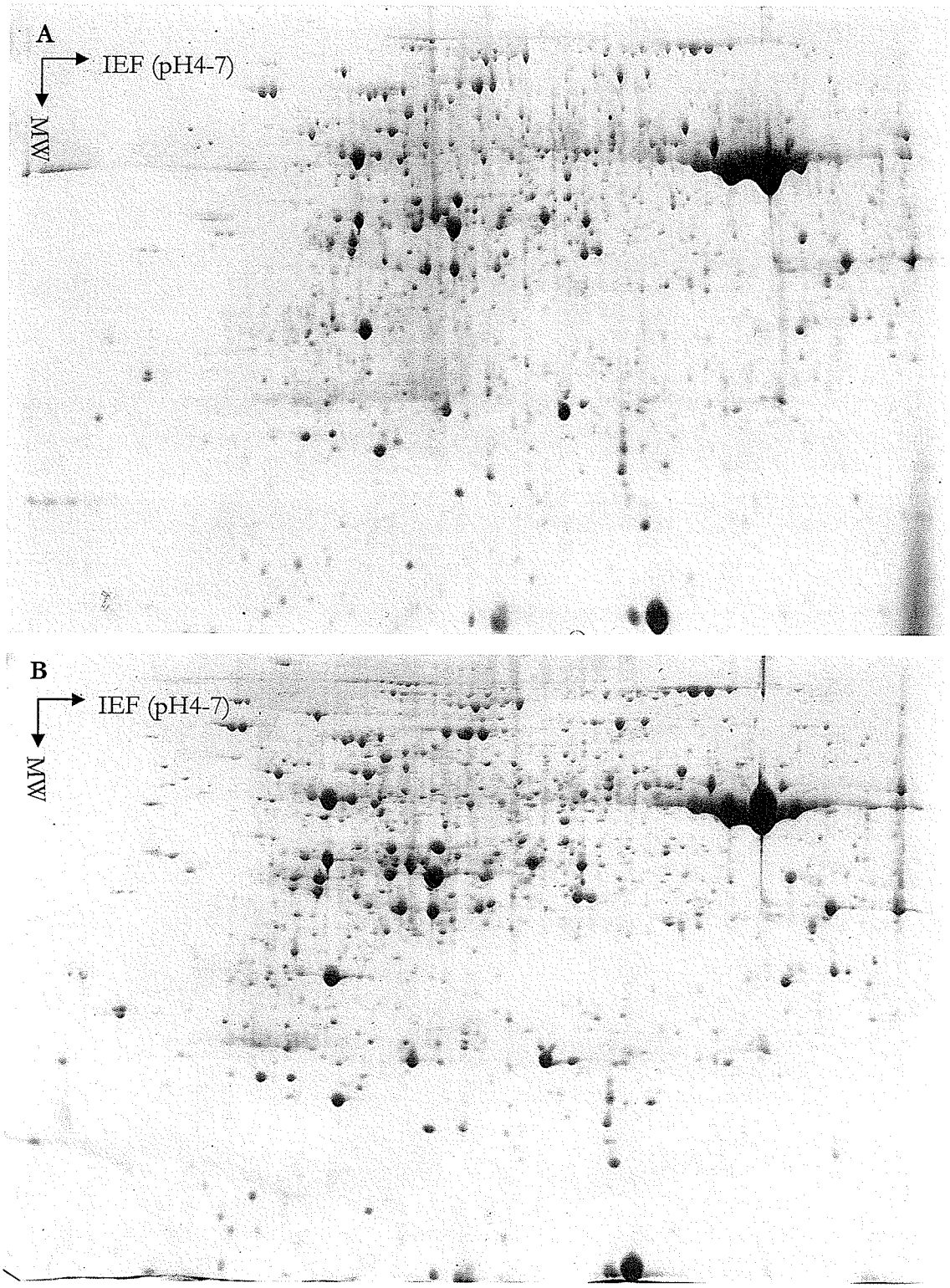


Figure 10. 2-DE separation of total soluble protein from Glenlea wheat leaves 2 days post infiltration with Ptr ToxA. Protein from extracted Glenlea wheat leaves infiltrated with 1:50 dilution of 40μg/μl of Ptr ToxA, 2 days post infiltration were resolved on pH 4-7 strips in the first dimension and separated on 12% SDS PAGE in the second dimension, and stained with Coomassie brilliant blue. (A) biological control replicate 1 and (B) biological control replicate 2.

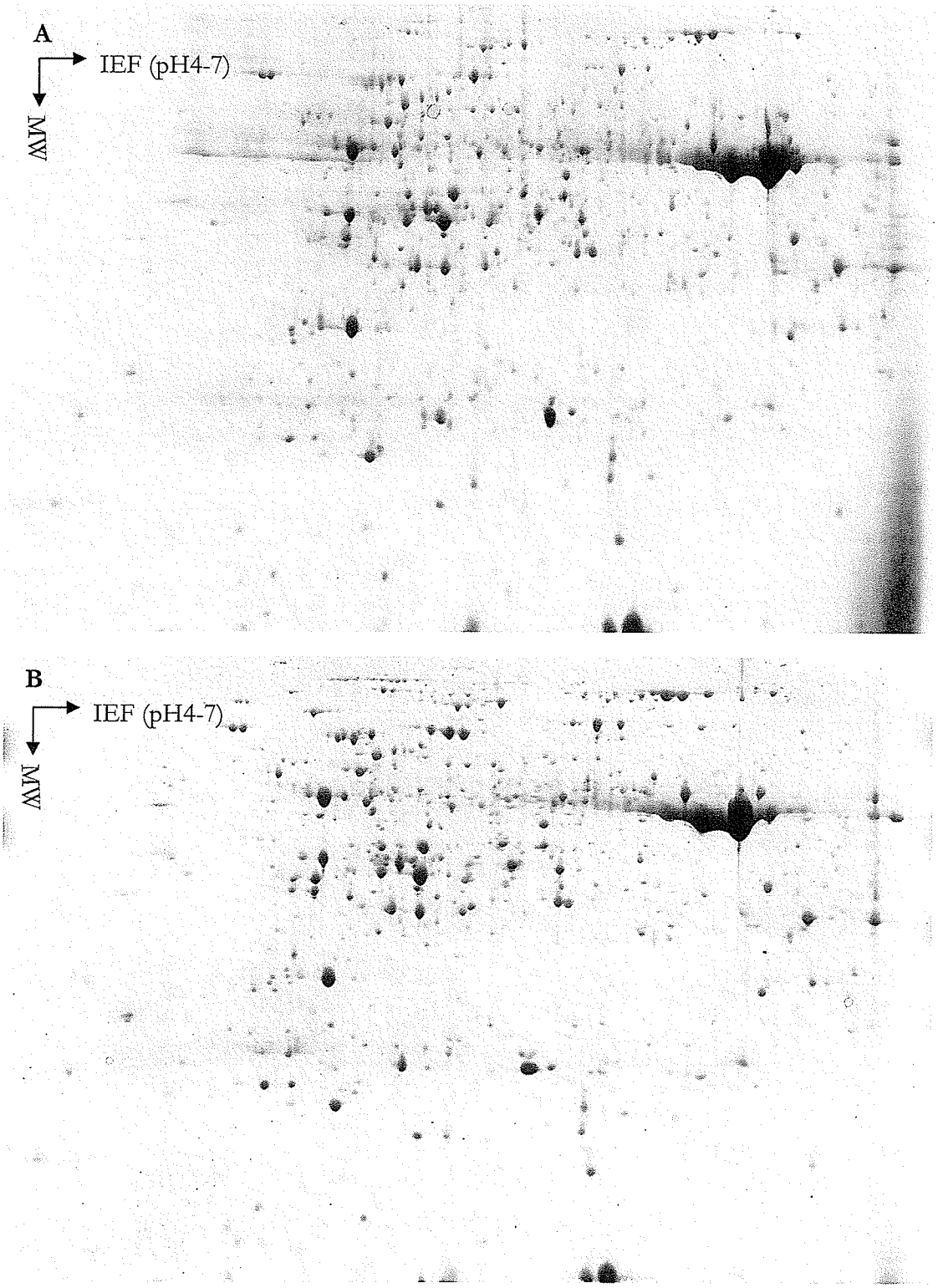


Figure 11. 2-DE separation of total soluble protein from Glenlea wheat leaves 3 days post infiltration with Ptr ToxA. Protein from extracted Glenlea wheat leaves infiltrated with a 1:50 dilution of 40μg/μl of Ptr ToxA, 3 days post infiltration were resolved on pH 4-7 strips in the first dimension and separated on 12% SDS PAGE in the second dimension, and stained with Coomassie brilliant blue. (A) biological control replicate 1 and (B) biological control replicate 2.

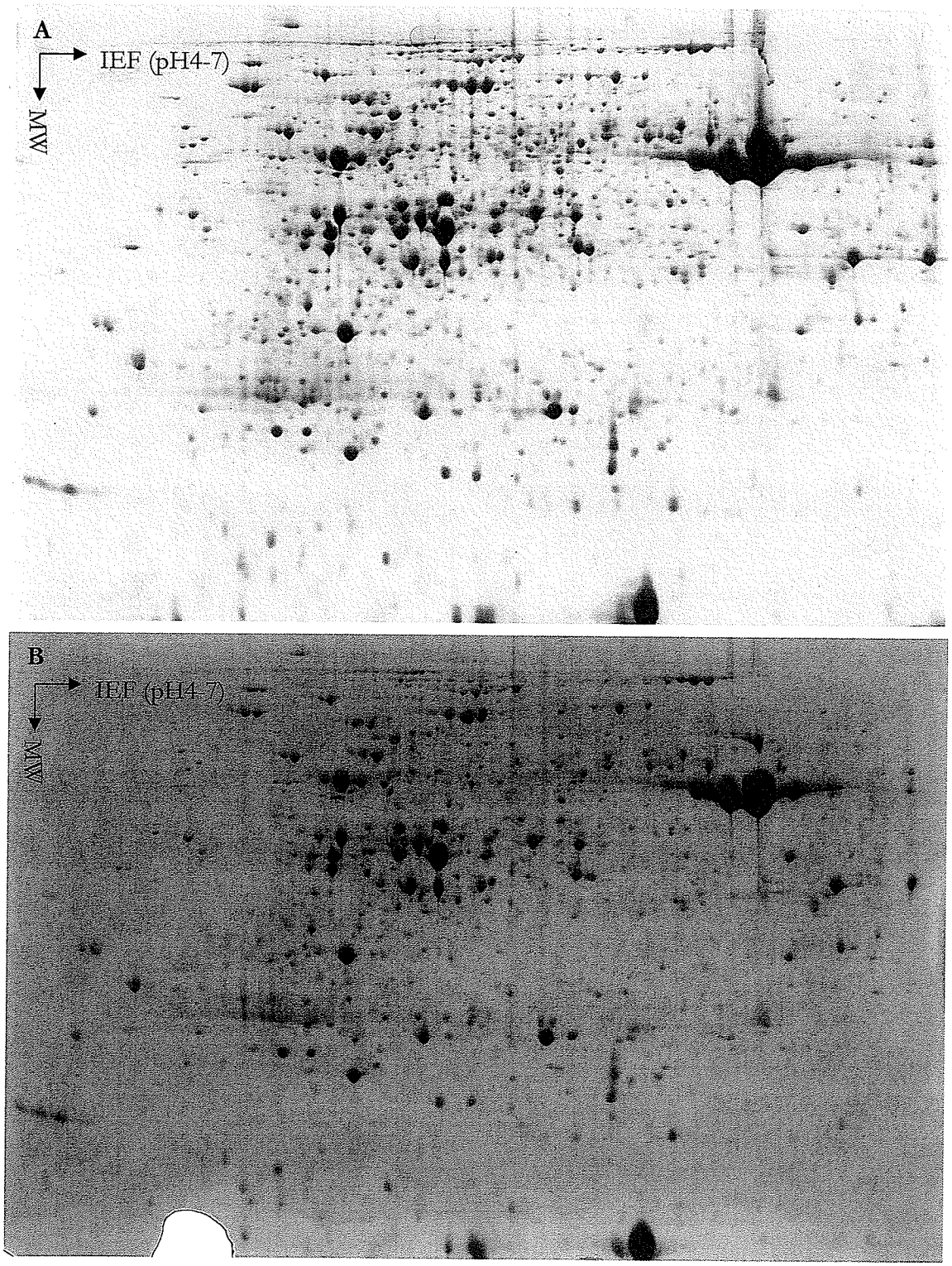


Figure 12. 2-DE separation of total soluble protein from Amazon wheat leaves 1 day post infiltration with control buffer. Protein from extracted Amazon wheat leaves infiltrated with 1:50 dilution of 10mM sodium acetate pH 4.0, 1 day post infiltration were resolved on pH 4-7 strips in the first dimension and separated on 12% SDS PAGE in the second dimension, and stained with Coomassie brilliant blue. (A) biological control replicate 1 and (B) biological control replicate 2.

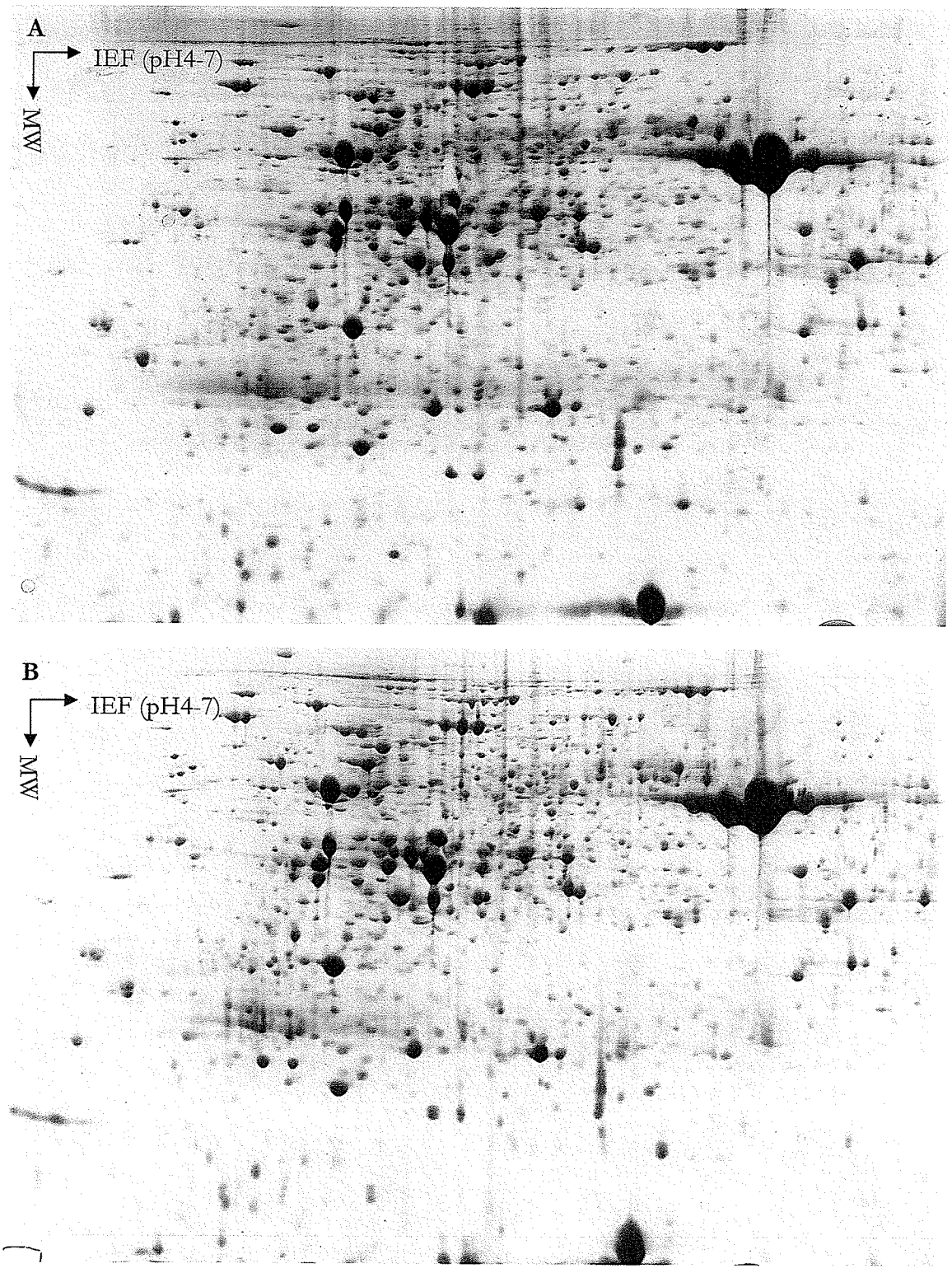


Figure 13. 2-DE separation of total soluble protein from Amazon wheat leaves 2 days post infiltration with control buffer. Protein from extracted Amazon wheat leaves infiltrated with 1:50 dilution of 10mM sodium acetate pH 4.0, 2 days post infiltration were resolved on pH 4-7 strips in the first dimension and separated on 12% SDS PAGE in the second dimension, and stained with Coomassie brilliant blue. (A) biological control replicate 1 and (B) biological control replicate 2.

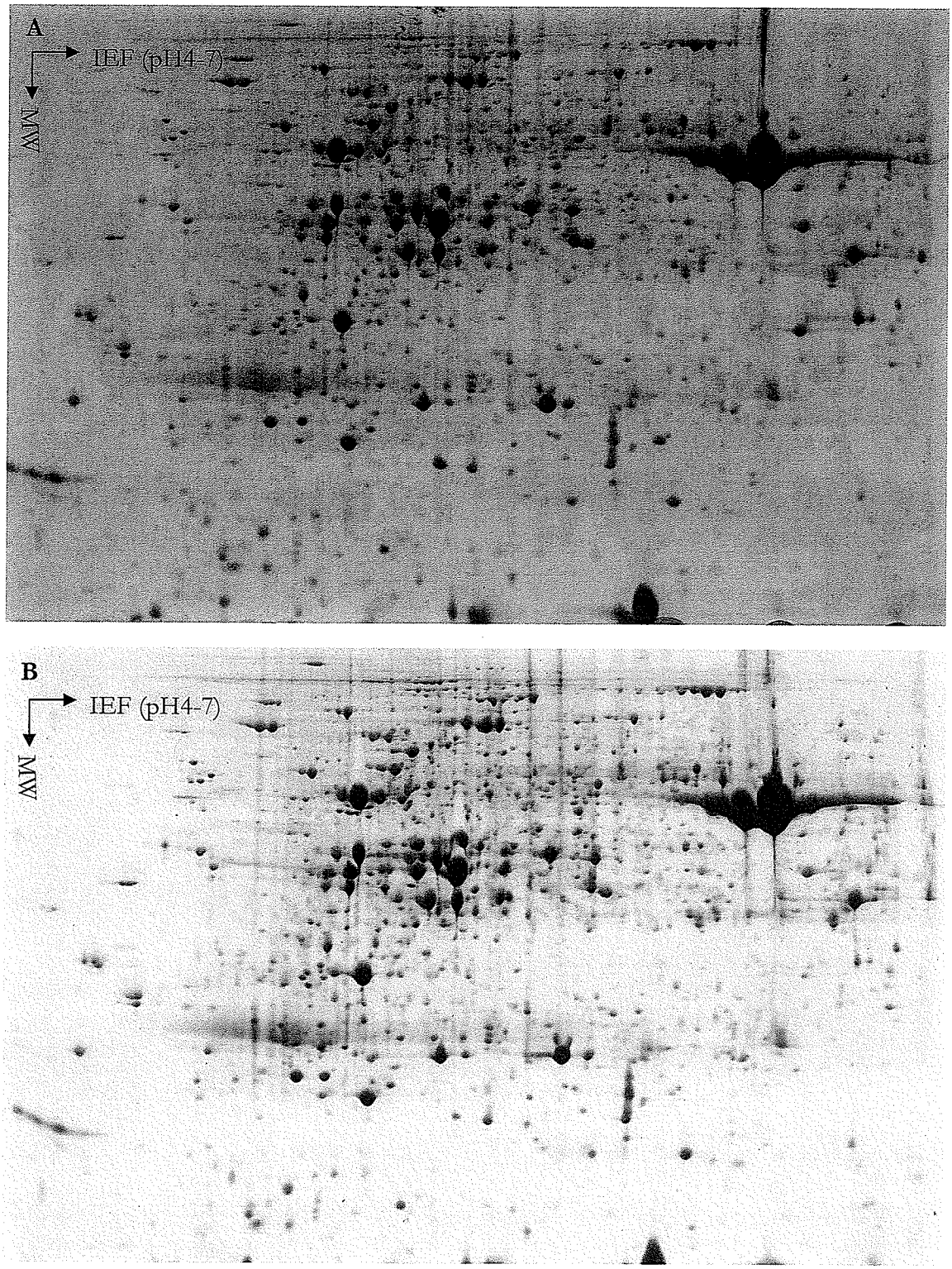


Figure 14. 2-DE separation of total soluble protein from Amazon wheat leaves 3 days post infiltration with control buffer. Protein from extracted Amazon wheat leaves infiltrated with 1:50 dilution of 10mM sodium acetate pH 4.0, 3 days post infiltration were resolved on pH 4-7 strips in the first dimension and separated on 12% SDS PAGE in the second dimension, and stained with Coomassie brilliant blue. (A) biological control replicate 1 and (B) biological control replicate 2.

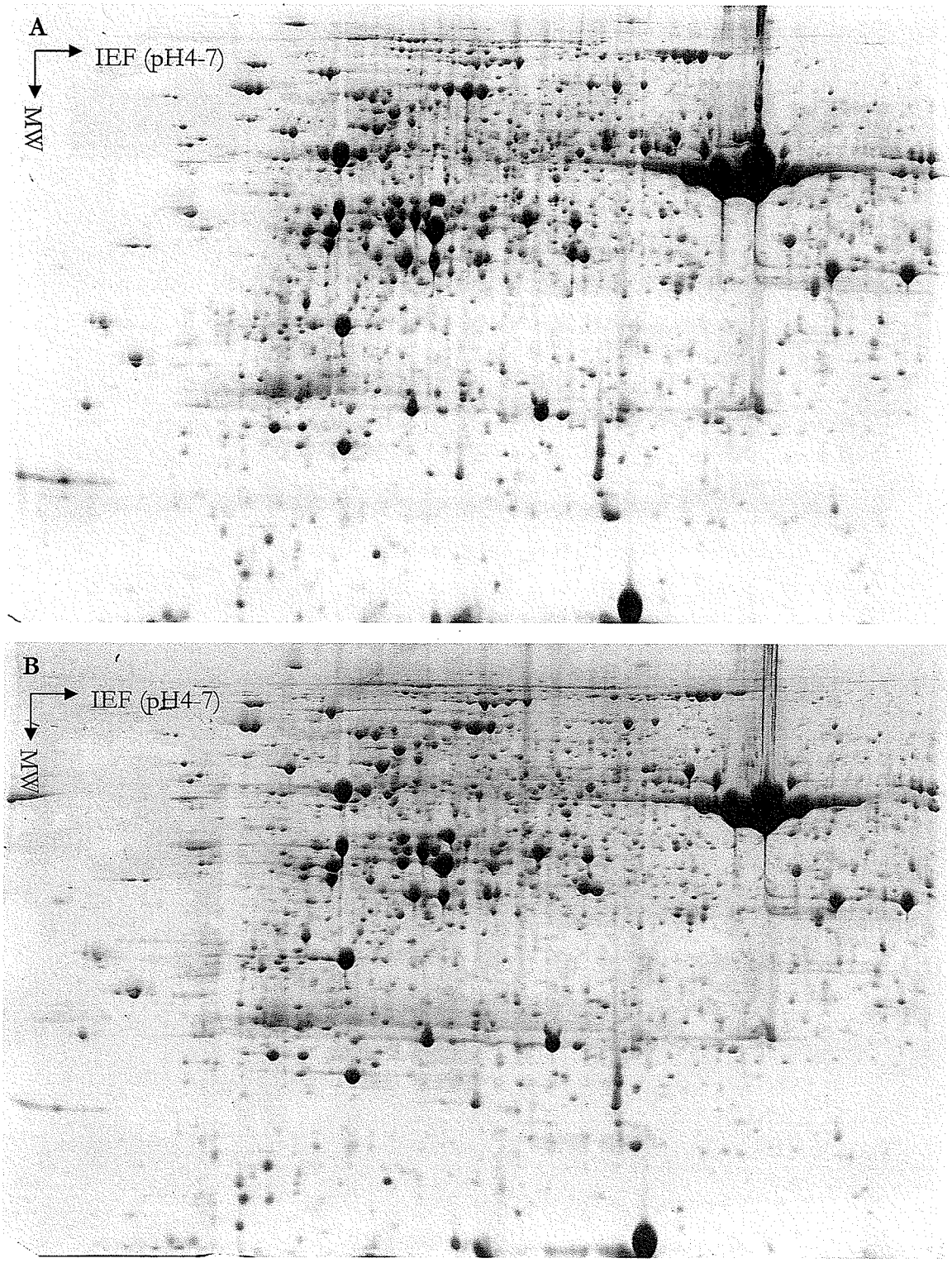


Figure 15. 2-DE separation of total soluble protein from Amazon wheat leaves 1 day post infiltration with Ptr ToxA. Protein from extracted Amazon wheat leaves infiltrated with a 1:50 dilution of 40µg/µl of Ptr ToxA, 1 day post infiltration were resolved on pH 4-7 strips in the first dimension and separated on 12% SDS PAGE in the second dimension, and stained with Coomassie brilliant blue. (A) biological control replicate 1 and (B) biological control replicate 2.

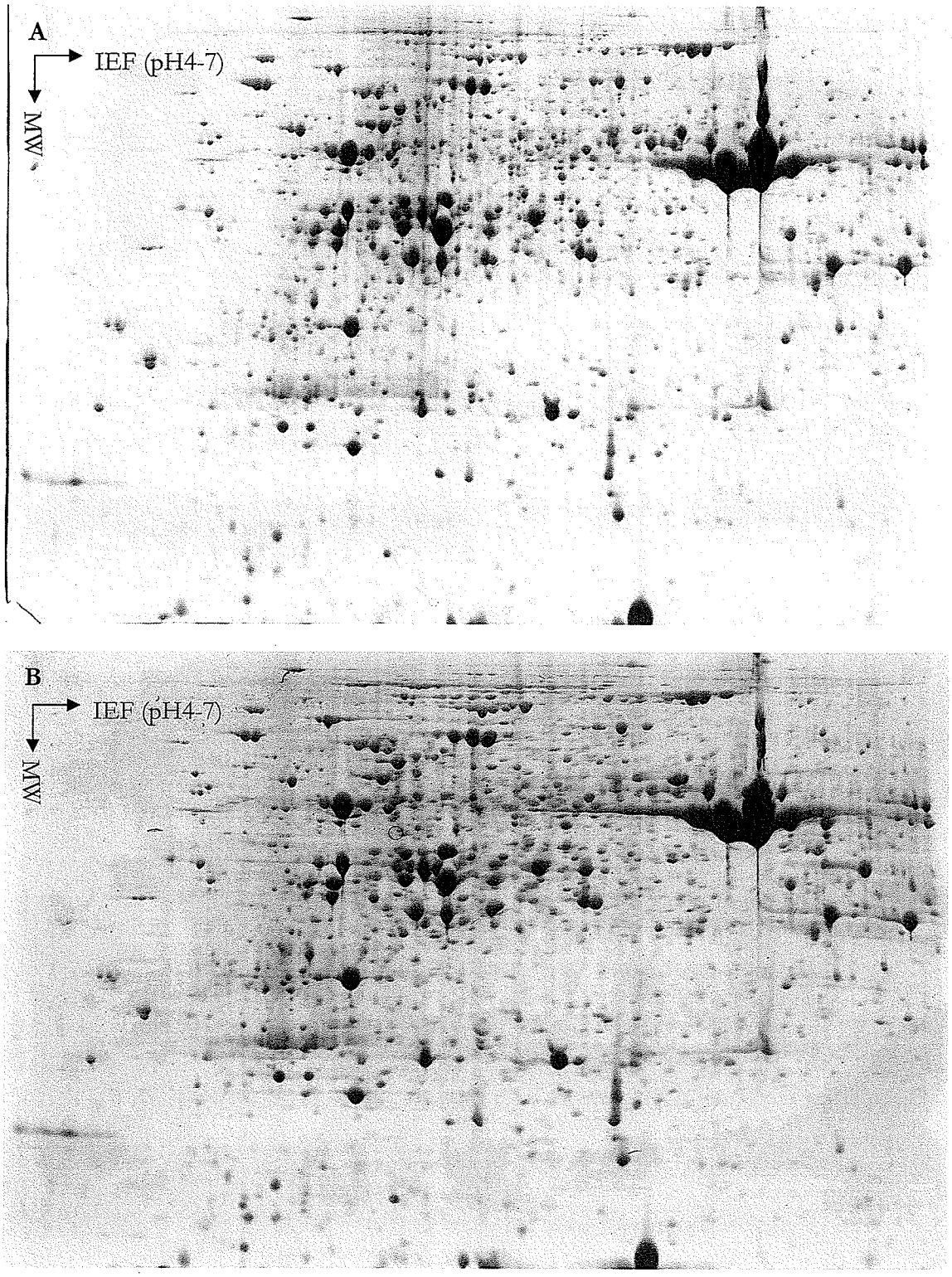


Figure 16. 2-DE separation of total soluble protein from Amazon wheat leaves 2 days post infiltration with Ptr ToxA. Protein from extracted Amazon wheat leaves infiltrated with a 1:50 dilution of 40μg/μl of Ptr ToxA, 2 days post infiltration were resolved on pH 4-7 strips in the first dimension and separated on 12% SDS PAGE in the second dimension, and stained with Coomassie brilliant blue. (A) biological control replicate 1 and (B) biological control replicate 2.

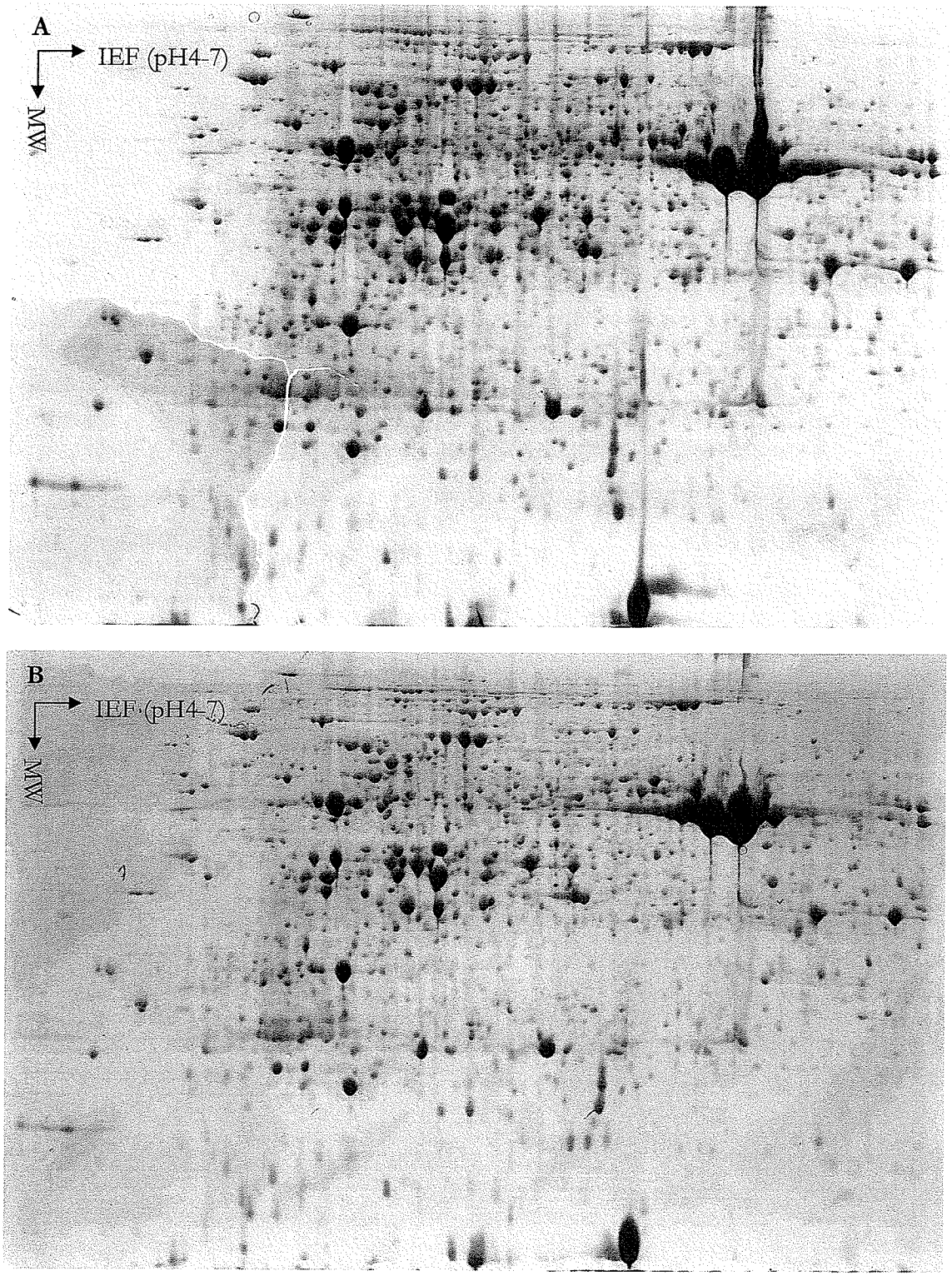
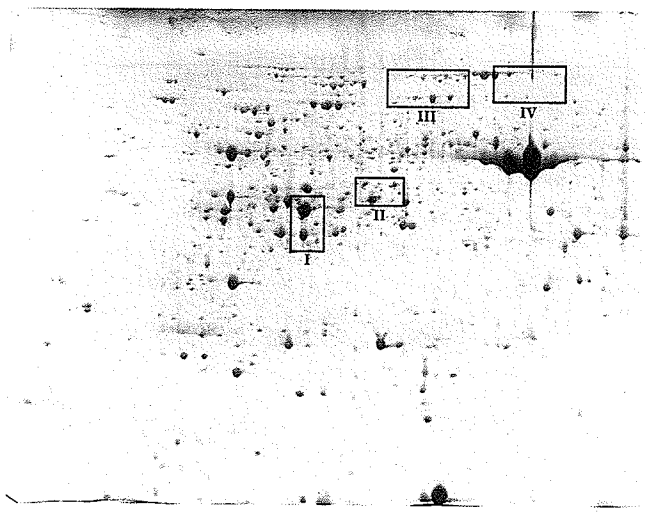
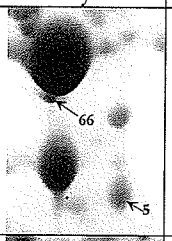
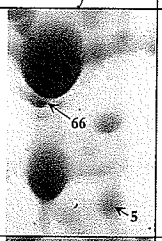
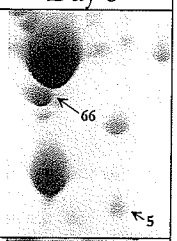
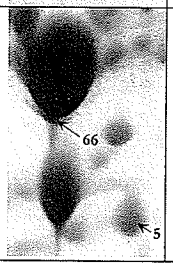
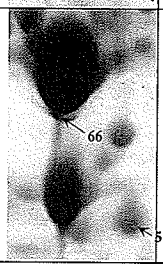
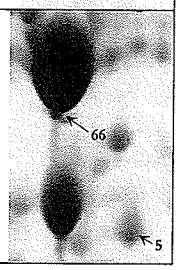
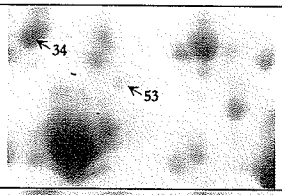
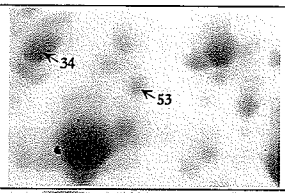
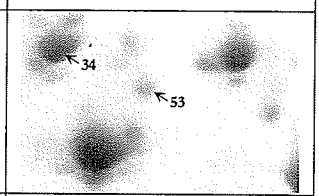
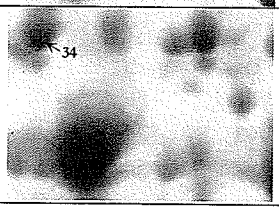
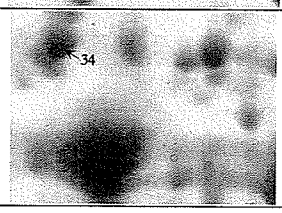
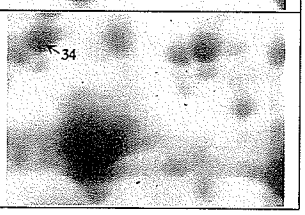


Figure 17. 2-DE separation of total soluble protein from Amazon wheat leaves 3 days post infiltration with Ptr ToxA. Protein from extracted Amazon wheat leaves infiltrated with a 1:50 dilution of 40μg/μl of Ptr ToxA, 3 days post infiltration were resolved on pH 4-7 strips in the first dimension and separated on 12% SDS PAGE in the second dimension, and stained with Coomassie brilliant blue. (A) biological control replicate 1 and (B) biological control replicate 2.



I	Day 1	Day 2	Day 3
toxin			
control			

II	Day 1	Day 2	Day 3
toxin			
control			

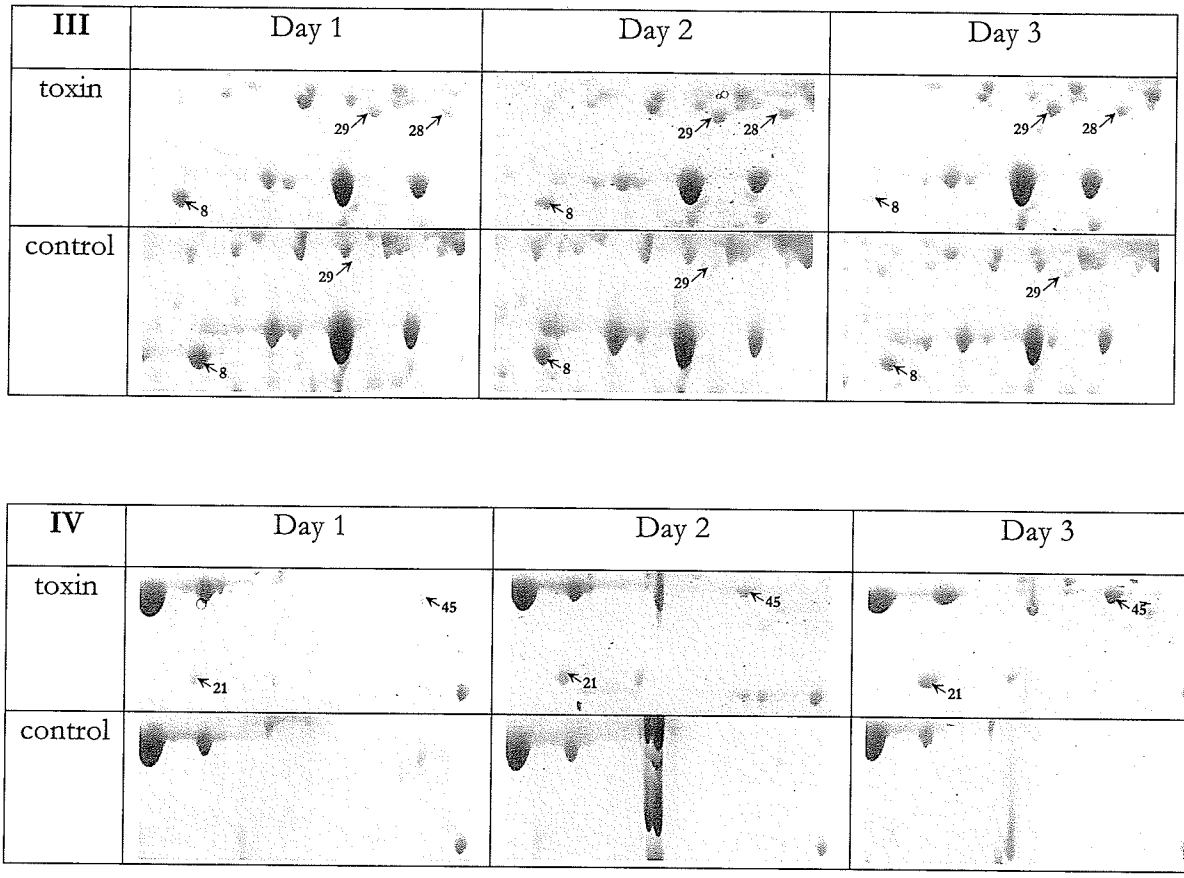
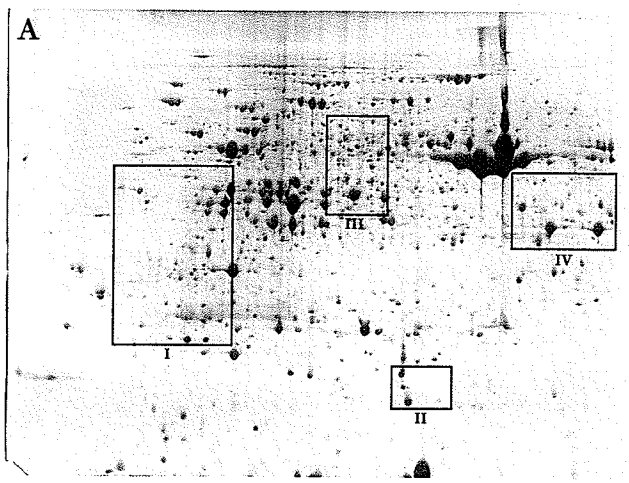
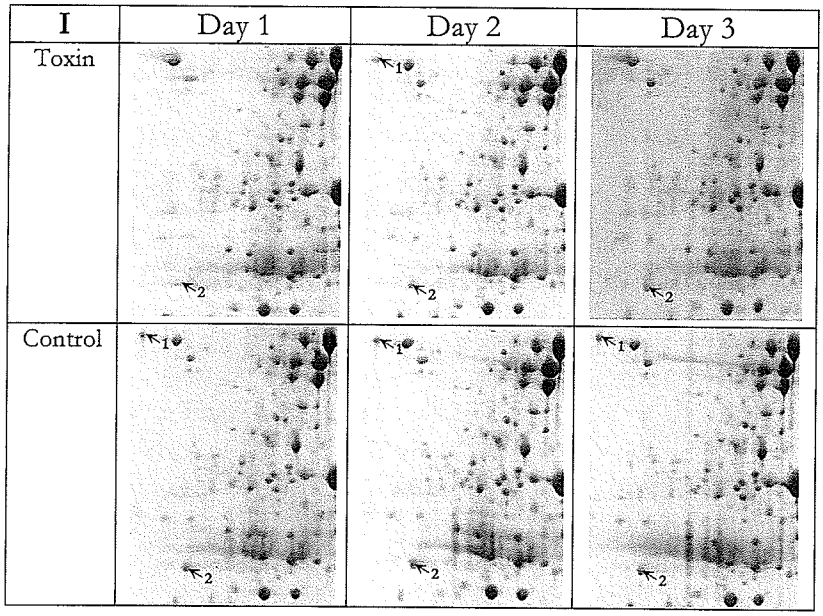
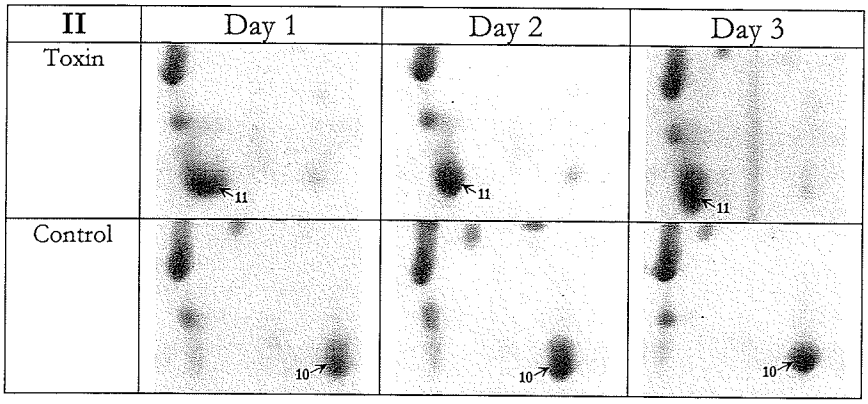
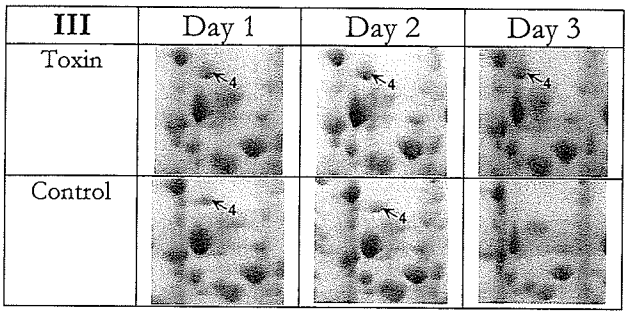


Figure 18. Protein Changes in 2D electrophoresis of Glenlea wheat leaves infiltrated with Ptr ToxA and control buffer. Regions of protein changes depicted in the representative Glenlea 2D gel (A) were magnified in tables below the gel. Expression of spot 66 (I), spot 34 (II), and spot 29 (III) progressively increased throughout day 1-3 in the toxin treated plants and remained relatively constant in the control however, spot 5 (I) and spot 8 (III) progressively decreased in toxin treated samples. Spot 53 (II), spot 28 (III), spot 45 and 21 (IV) appeared only in Glenlea leaves treated with toxin and showed an increasing expression from day 1 to day 3.


A
III
IV
I
II


I
Day 1
Day 2
Day 3
Toxin
Control
1
2


II
Day 1
Day 2
Day 3
Toxin
Control
11
10



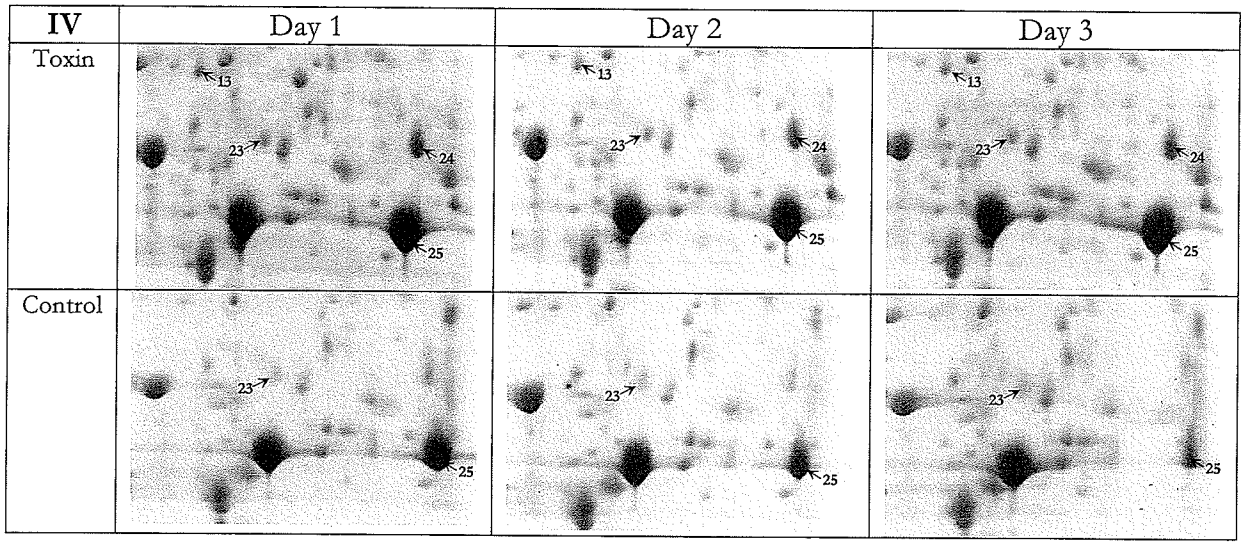


Figure 19. Protein Changes in 2D electrophoresis of Amazon wheat leaves infiltrated with Ptr ToxA and control buffer. Regions of protein changes depicted in the representative Amazon 2D gel (A) were magnified in tables below. Spot 1 was not present in day 1 or 3 but present in Day 2 of the toxin treated plants, while remaining relatively constant in the control (I) and spot 2 showed a general decreased expression in comparison to the control (I). Spot 11 appeared in the toxin treated samples and not the control while spot 10 existed only in the control (II). Spot 13 and 24 were present only in the toxin treated leaves while spot4 (III), spots 23 and 25 (IV) showed an upregulation in treated leaves.

Table 2: Identification of upregulated and downregulated proteins in Glenlea leaves infiltrated with Ptr ToxA using MASCOT identification with MS/MS spectra

Spot # [a]	Putative Identification	Identification	Taxonomy	MS/MS MASCOT [b]	Score [c]	Percent Coverage
5	Ferredoxin-NADP(H) oxidoreductase	gi 20322473	*Triticum aestivum*	**K.KQDEGVVTNK** **K.QDEGVVTNK** **K.EPYVGR** **R.CLLNTR** **R.LTGDNAPGETWHMVFSTEGEVPYR** **R.EGQSIGVIADGEDK** **R.EGQSIGVIADGEDKNGKPHK** **R.LYSIASSALGDFGDSK** **K.TVSLCVK** **K.RLVYTNDAGEVVK** **R.LVYTNDAGEVVK** **K.GVCSNFLCDLKPGSEVK** **K.DPNATIIMLATGTGIAPFR** **R.SFLWK** **K.MFFEEHEDYK** **K.EEFEK** **K.MVEIGGENFR** **R.LDFAVSR** **R.MAEYK** **R.MAEYKEELWEMLK** **K.KDNTYVYMCGLK** **K.DNTYVYMCGLK** **K.GIDDIMVDLAAK** **K.DGIDWIDYK** **K.KAEQWNVEVY** **TOTAL**	55 30 25 26 72 83 64 114 11 65 73 61 111 25 24 24 54 23 10 63 28 28 93 36 29 1278	**69%**
8	putative GTP-binding protein typA	gi 50906979	*Oryza sativa*	**R.NIAIVAHVDHGK** **K.VFRDNQVVQER** **R.IMDSNDLER** **R.GITILSK**	67 26 25 28	**35%**

				K.NTSITYK	51	
				K.INIIDTPGHSDFGGEVER	89	
				K.ALEFGHAVVVVVNK	107	
				R.CIPEPR	39	
				K.VCTPDDACR	19	
				K.IEEPTVR	24	
				R.MSFSINTSPFVGK	62	
				R.VEDGETADTFLVSGR	95	
				R.GTLHLTILIENMR	57	
				K.LQEPYEIAAVEVPEEYMGSVVELLGK	30	
				K.GQIVGGIHQRPGDLAINVCK	65	
				TOTAL	784	
28	ATP-dependant Clp protease ATP-binding subunit precursor	gi 26518520	*Oryza sativa*	R.EEEIER	23	20%
				R.VVQIICR	16	
				K.GLGEGAVAPR	20	
				K.SLVMEELK	23	
				K.TAIAEGLALR	18	
				K.AIDLIDEAGSR	75	
				R.LDMSEYMER	20	
				K.NNPILLGEAGVGK	61	
				R.SAGGFLSSGINIER	28	
				R.TKNNPILLGEAGVGK	44	
				R.KPFTVVLLDEIEK	51	
				K.ALAASYFGSESAMLR	81	
				K.FTLEAINAAVYLSAR	83	
				R.RKPFTVVLLDEIEK	37	
				R.GELQCIAATTLDEHR	71	
				R.QLPDKAIDLIDEAGSR	47	
				K.LIGSPPGYIGYGETGTLTEAVR	34	
				TOTAL	732	
29	ATP-dependant Clp protease ATP-binding subunit precursor	gi 26518520	*Oryza sativa*	K.ILLGLR	19	21%
				R.VVQIICR	19	
				K.GLGEGAVAPR	29	
				K.TAIAEGLALR	28	
				K.AIDLIDEAGSR	39	
				K.NNPILLGEAGVGK	59	
				R.KPFTVVLLDEIEK	68	
				K.ALAASYFGSESAMLR	71	

				K.FTLEAINAAVYLSAR R.RKPFTVVLLDEIEK R.GELQCIAATTLDEHR K.LIGSPPGYIGYGETGTLTEAVR K.GALDQFCLDLTTQASGGFIDPIIGREEEIER TOTAL	94 42 81 82 100 731	
21	Methionine synthase 2 enzyme	gi 68655500	*Hordeum vulgare*	R.NDQPR R.EYKAK R.EGLPLR K.AVSEYK K.LDSEIK R.TQLASTK K.FSYASHK K.QMSEAGIK K.SSAEDLEK K.VVEVNALGK M.ASHIVGYPR K.SWLAFAAQK K.YLFAGVVDGR R.IPSTEEIADR K.FALESFWDGK R.GNATVPAMEMTK R.FETCYQIALAIK R.IPSTEEIADRVNK K.DEAYFAANAAAQASR K.AAGASWIQFDEPTLVK K.TLTSLSGVTAYGFDLVR K.GMLTGPVTILNWSFVR R.KYTEVKPALTNMVAAAK K.SEHAFYLDWAVHSFR R.KSEHAFYLDWAVHSFR K.IQEELDIDVLVHGEPER R.EGVTYGAGIGPGVYDIHSPR R.YSWTGGEIGHSTYFSMAR K.ALAGQKDEAYFAANAAAAQASR K.ALGVDTVPVLVGPVSYLLLSK K.WFDTNYHFIVPELSPATK K.MLAVLDTNILWVNPDCGLK	27 6 20 16 19 35 30 17 29 44 13 44 58 68 56 16 79 52 89 73 105 116 55 87 51 115 88 70 116 68 60 85	65%

				K.EVEDLEAGGIQVIQIDEAALR K.LVVSTSCSLMHTAVDLVNETK K.DKLVVSTSCSLMHTAVDLVNETK R.CVKPPIIYGDVSRPNPMTVFWSK K.YIPSNTFSYYDQVLDTTAMLGAVPDR K.LVVSTSCSLMHTAVDLVNETKLDSEIK TOTAL	123 89 31 24 69 22 2165	
34	putative Ado Met synthase 3	gi 68655446	*Hordeum vulgare*	R.LTEVR R.SGAYIAR K.ATVDYEK K.SIIASGLAR K.VACETVTK K.TAAYGHFGR K.EHVIKPVIPAK K.NGTCAWVRPDGK R.FVIGGPHGDAGLTGR K.TNMVMVLGEITTK R.KNGTCAWVRPDGK R.NIGFISDDVGLDADR R.DDADFTWEVVKPLK K.YLDENTIFHLNPSGR K.ENFDFRPGMISINLDLK K.TQVTVEYLNEDGAMVPVR R.DDADFTWEVVKPLKFDK R.KIIIDTYGGWGAHGGGAFSGK K.ENFDFRPGMISINLDLKK K.LCDQVSDAVLDACLAQDADSK K.VLVNIEQQSPDIAQGVHGHFTK K.TAAYGHFGRDDADFTWEVVKPLK R.VHTVLISTQHDETVTNDEIAADLK R.CIVQISYAIGVPEPLSVFVDSYGTGK R.RCIVQISYAIGVPEPLSVFVDSYGTGK TOTAL	20 24 26 26 28 16 50 46 95 103 27 122 67 77 43 114 12 88 36 123 95 59 90 48 40 1475	77%
45	Heat shock protein 101	gi 6013196	*Triticum aestivum*	R.HLPDK R.LTDGQGR R.VKAEVEK K.EVEEAEGK R.WTGIPVTR	15 20 20 35 16	10%

				K.YRGEFEER	24	
				K.NNPVLIGEPGVGK	48	
				R.TKNNPVLIGEPGVGK	34	
				R.VQLDSQPEEIDNLER	63	
				TOTAL	275	
53	Cinnamyl alcohol dehydrogenase 2a	gi 15428280	*Festuca arundinacea*	K.GLTSQIEVVK	64	51%
				K.ANVEQYCNK	29	
				K.HFGLMTPGLR	38	
				K.ANVEQYCNKK	23	
				K.HPLEPYLALLK	47	
				R.GGILGLGGVGHMGVK	41	
				K.SMGHHVTVISSSNK	48	
				K.YPMVPGHEVVGEVVEVGPEVSK	61	
				K.LVLMGVIAEPLSFVSPMVMLGR	69	
				K.IPAGLAPEQAAPLLCAGVTVYSPLK	85	
				K.IWSYNDVYTDGKPTQGGFASAMVVDQK	76	
				K.KIWSYNDVYTDGKPTQGGFASAMVVDQK	52	
				TOTAL	633	
66	Glutamine synthase isoform GS1b	gi 71361902	*Triticum aestivum*	R.YLLER	19	71%
				K.VIVDAVEK	33	
				R.DIVDSHYK	52	
				K.IFSNPDVAK	32	
				R.TLPGPVTDPSK	37	
				K.EHIAAYGEGNER	65	
				K.EHIAAYGEGNERR	26	
				R.ETEQNGKGYFEDR	30	
				K.HKEHIAAYGEGNER	62	
				K.IIAEYIWIGGSGMDLR	100	
				K.HETADINTFSWGVANR	100	
				M.ALLTDLLNLDLTDSTEK	98	
				K.EEPWYGIEQEYTLLQK	64	
				R.LTGKHETADINTFSWGVANR	65	
				K.GNNILVMCDCYTPAGVPIPTNK	71	
				R.KGNNILVMCDCYTPAGVPIPTNK	38	
				K.GNNILVMCDCYTPAGVPIPTNKR	55	
				K.WNYDGSSTGQAPGEDSEVILYPQAIFK	92	
				K.IFSNPDVAKEEPWYGIEQEYTLLQK	82	
				K.DINWPLGWPVGGFPGPQGPYYCSIGADK	58	

				K.WNYDGSSTGQAPGEDSEVILYPQAIFKDPFR	77	
				TOTAL	1256	

(a) Spot numbers correspond to number in Figure 18.
(b) Matched peptide sequences in identified proteins by MASCOT MS/MS ion search
(c) MASCOT Ions score are -10*Log(P), where P is the probability that the observed match is a random event. Individual ions scores > 47 indicate identity or extensive homology ($p<0.05$).

Table 3: Identification of upregulated and downregulated proteins in Amazon leaves infiltrated with Ptr ToxA using MASCOT identification with MS/MS spectra

Spot # [a]	Putative Identification	Identification	Taxonomy	MS/MS MASCOT [b]	Score [c]	Percent Coverage
13	elongation factor 1 gamma-like protein	gi 29367403	*Oryza sativa*	**K.MNPIGK** **R.KYAFGK** **R.LYSNTK** **K.TPEFLK** **K.WLYPR** **K.VDISDEAQK** **K.NFQMGVSNK** **K.NPLDLLPPSK** **K.VELVKNFQMGVSNK** **R.YFWTMVNQPNFKK** **K.NFQMGVSNKTPEFLK** **TOTAL**	18 27 34 24 15 42 22 57 24 28 49 340	28%
2	Type III LHCII CAB precursor protein	gi 19023	*Hordeum vulgare*	**K.EPVWFK** **K.WVGVEFK** **R.ALEVIHGR** **R.NRALEVIHGR** **K.WVGVEFKEPVWFK** **R.WAMLGALGCVFPEVLQK** **R.LAMFSMFGFFVQAIVTGK** **K.GPLENLFDHLDDPVANNAWVFATK** **TOTAL**	25 26 21 41 28 73 96 55 365	32%
4	putative methylenetetrahydrofolate reductase	gi 50919385	*Oryza sativa*	**K.SYISR** **R.MTGFCK** **K.SVEDINER** **K.SNGIQNVLALR** **K.AFPSLTYIAVNK** **K.TLHLYTLNMEK** **K.AYGIHLGTEMCKK** **K.SKAFPSLTYIAVNK** **R.VKEDVRPIFWANRPK**	12 21 19 15 27 33 32 62 25	33%

				R.QIGITCPIVPGIMPINNYK R.TVFSFEYFPPKTEEGLDNLFER K.GFLTINSQPAVNGERSDSTSVGWGGPGGYVY QK TOTAL	49 25 34 354	
1	Triticain beta [1]	gi 111073717	*Triticum aestivum*	K.DYWIVR K.AVAHQPVSVAIEAGGR TOTAL	24 29 53	19%
23	Putative hydroxypyruvate reductase	gi 50904581	*Oryza sativa*	K.QLGLPSSK K.FVTAYGQFLK R.EADVISLHPVLDK R.EADVISLHPVLDKTTYHLINPER TOTAL	40 22 43 33 138	16%
10	putative Rieske Fe-S precursor protein	gi 32394644	*Triticum aestivum*	R.VPDMSK R.TLAQGLK K.THGPNDR R.TGDNPWWK K.GDPTYLVVESDK K.LGNDILVEDWLK K.VVFVPWVETDFR K.THGPNDRTLAQGLK K.DKLGNDILVEDWLK R.GPAPLSLALVHADVDDGK K.FLCPCHGSQYNNQGK R.TLAQGLKGDPTYLVVESDK K.TLATYGINAVCTHLGCVVPWNAAENK R.GPAPLSLALVHADVDDGKVVFVPWVETDFR TOTAL	24 21 19 39 68 76 40 43 90 97 67 76 99 79 838	56%
11	putative Rieske Fe-S precursor protein	gi 32394644	*Triticum aestivum*	R.TLAQGLK K.THGPNDR R.TGDNPWWK K.GDPTYLVVESDK K.LGNDILVEDWLK K.VVFVPWVETDFR K.THGPNDRTLAQGLK K.DKLGNDILVEDWLK R.GPAPLSLALVHADVDDGK K.FLCPCHGSQYNNQGK	18 13 26 46 73 19 15 91 80 58	53%

				R.TLAQGLKGDPTYLVVESDK K.TLATYGINAVCTHLGCVVPWNAAENK R.GPAPLSLALVHADVDDGKVVFVPWVETDFR TOTAL	89 79 67 674	
24	putative hydroxypyruvate reductase	gi 50904581	*Oryza sativa*	K.ANPMFR K.QLGLPSSK K.QLGLPSSKL K.EAVLVNASR K.GQTVGVIGAGR R.IVEADQFMR K.FVTAYGQFLK K.TTYHLINPER R.RIVEADQFMR K.NAVVVPHIASASK R.EGMATLAALNVLGK R.EADVISLHPVLDK R.AGLYDGWLPHLFVGNLLK K.FVTAYGQFLKANGEQPVTWK R.VGLDVFEDEPYMKPGLADMK R.MMIEGFKMNLIYYDLYQSTR R.EADVISLHPVLDKTTYHLINPER TOTAL	8 40 37 52 35 28 37 34 48 48 78 82 34 17 20 29 84 711	48%
25	Glyceraldehyde-3-phosphate dehydrogenase A[2]	gi 120657	*Zea mays*	K.LNGIALR K.VLITAPGK R.LLDASHR K.VAINGFGR K.KVLITAPGK K.GKLNGIALR K.AVALVLPNLK K.TLAEEVNQAFR R.AAALNIVPTSTGAAK K.KTLAEEVNQAFR R.VVDLADIVANQWK K.GTMTTTHSYTGDQR R.VPTPNVSVVDLVVQVSK K.VIAWYDNEWGYSQR R.VPTPNVSVVDLVVQVSKK K.FGIIKGTMTTTHSYTGDQR	23 35 21 21 45 31 50 65 63 79 81 81 116 75 118 24	54%

				K.YDSTLGIFDADVKPVGDNAISVDGK K.YDSTLGIFDADVKPVGDNAISVDGKVIK TOTAL	86 19 1033	

(a) Spot numbers correspond to number in Figure 19.
(b) Matched peptide sequences in identified proteins by MASCOT MS/MS ion search
(c) MASCOT Ions score are -10*Log(P), where P is the probability that the observed match is a random event. Individual ions scores > 47 indicate identity or extensive homology ($p<0.05$).
(1) The original identification was an unnamed protein product, gi 218183, from *Oryza sativa*, when queried using the BLAST algorithm, it was identified as triticain beta in *Triticum aestivum* with a 91% identity and an e value of 0.0 (NCBI, March 2007).
(2) The original identification was OSJNBa0036B21.24, gi 50924788, from *Oryza sativa*, when queried using the BLAST algorithm, it was identified as Glyceraldehyde-3-phosphate dehydrogenase A in *Oryza sativa* with a 92% identity and an e value of 0.0 (NCBI, March,2007).

aestivum, *Oryza sativa* and *Hordeum vulgare*. Interestingly, in Amazon, the same protein was identified for two separate spots and this occurred with spot 10 and 11 as well as with spot 23 and 24. Upon closer examination of these spots, it is evident that these proteins have the same molecular weight but different isoelectric points. Thus in Amazon leaves only seven proteins were observed to change between replicates.

Some of the proteins identified in Glenlea were metabolic proteins which were seen to be upregulated included methionine synthase, putative Ado Met synthase 3, and glutamine synthases. Clp protease ATP binding subunit was identified in two separate spots, one showing an upregulation and the other appeared only in toxin infiltrated samples. Two upregulated proteins previously shown to be upregulated during stress of the plants include cinnamyl alcohol dehydrogenase and heat shock protein 101. Ferredoxin –NADP oxidoreductase, an important component of photosynthesis, and putative GTP-binding protein typA were the only two proteins identified to be downregulated.

Both Amazon and Glenlea had a similar spread of upregulation, down regulation and induction of proteins as a result of toxin infiltration. Out of the seven proteins identified three of the proteins were chloroplast related, including glyceraldehyde-3-phosphate dehydrogenase, Rieske Fe-S, and LHC II CAB. Other proteins involved in protein synthesis such as methylenetetrahydrofolate reductase and elongation factor were also upregulated. Tritician beta was continually down regulated throughout day one and three and gross increase was observed with hydroxypyruvate reductase.

3.3 Proteomic Changes in Chloroplasts

The same method of comparison used with leaves of Glenlea and Amazon were used with chloroplasts. The 2DE gels were compared for changes within cultivars treated with toxin and control buffer as well as between each other (Figure 20& 21, Amazon, and Figure 22 & 23, Glenlea) however the only reproducible changes occurred between Glenlea chloroplast incubated with toxin and control buffer. Only four reproducible protein changes were observed and all displayed an increased expression as a result of incubation with Ptr ToxA (Figure 24). MASCOT was used to identify the proteins based on their mass spectra, created by MS/MS, and from this is was discovered that two of the spots with the same molecular weight but different isoelectric points resulted in the same protein identification, thus only three proteins were identified in the *in vitro* experiment of treated chloroplast suspensions (Table 4). All three proteins, hairpin binding protein 1, ATP synthase CF1 beta chain and chloroplastic aldolase were identified to be chloroplastic proteins and all were upregulated.

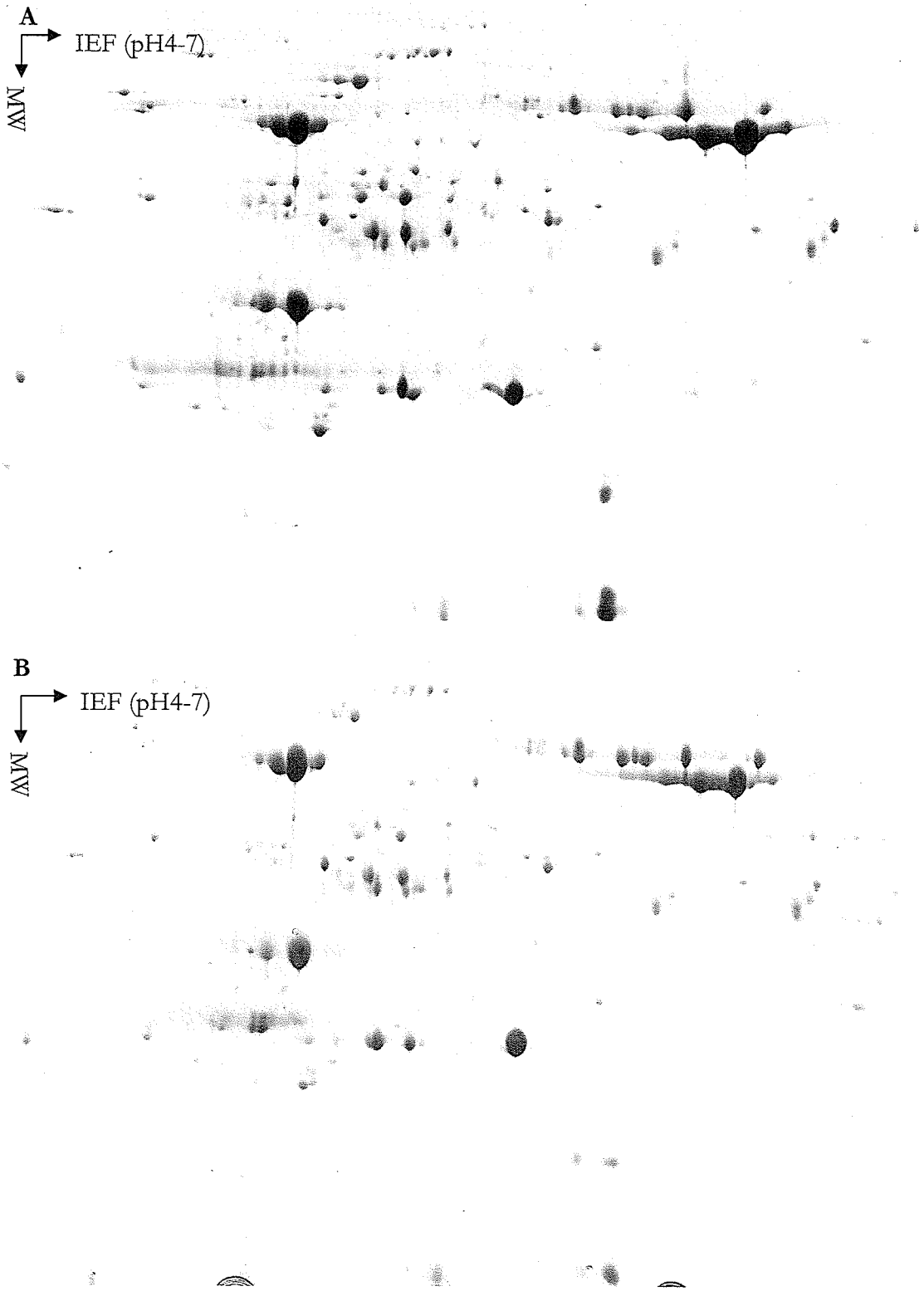


Figure 20. 2-DE separation of total soluble protein from Amazon chloroplast inoculated with control buffer. Isolated intact chloroplasts were inoculated with 10mM sodium acetate pH 4.0, for 1hr. Proteins extracted were resolved on pH 4-7 strips in the first dimension and separated on 12% SDS PAGE in the second dimension and stained with Coomassie brilliant blue.

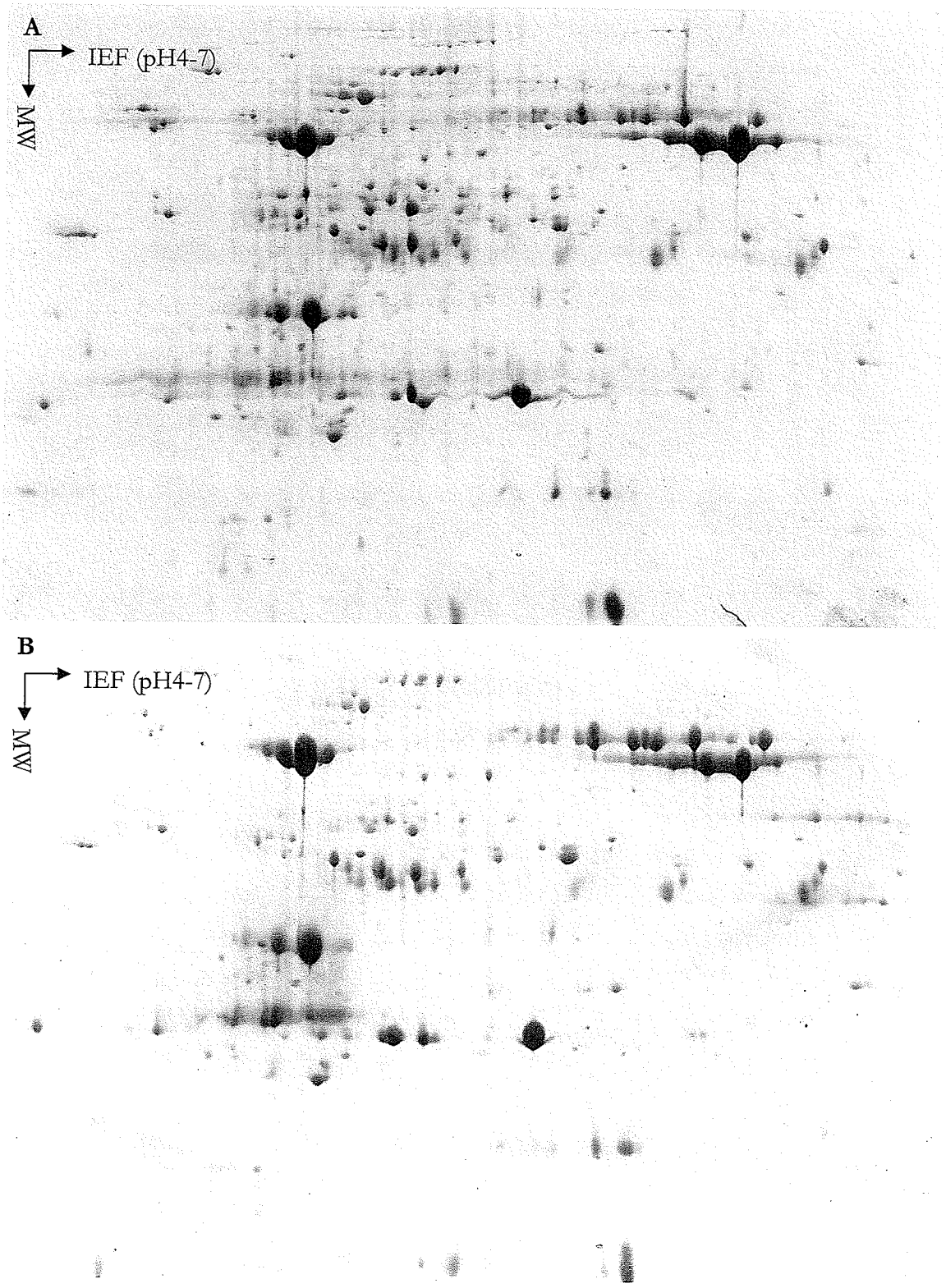


Figure 21. 2-DE separation of total soluble protein from Amazon chloroplast inoculated with Ptr ToxA. Isolated intact chloroplasts were inoculated with 1ng of Ptr ToxA for every 400,000 chloroplasts in solution, for 1 hr. Proteins extracted were resolved on pH 4-7 strips in the first dimension and separated on 12% SDS PAGE in the second dimension and stained with Coomassie brilliant blue.

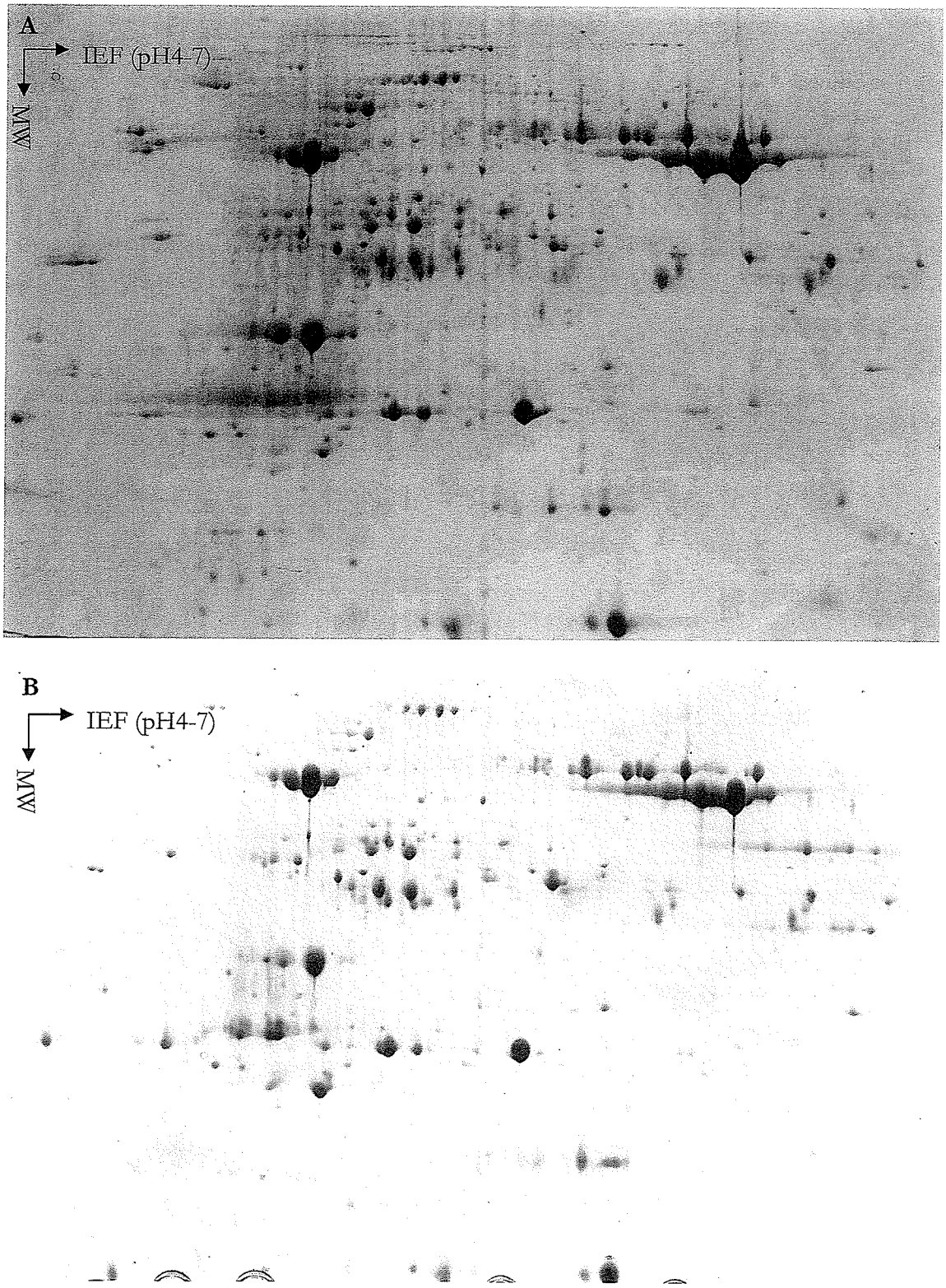


Figure 22. 2-DE separation of total soluble protein from Glenlea chloroplast inoculated with control buffer. Isolated intact chloroplasts were inoculated with 10mM sodium acetate pH 4.0, for 1hr. Proteins extracted were resolved on pH 4-7 strips in the first dimension and separated on 12% SDS PAGE in the second dimension and stained with Coomassie brilliant blue.

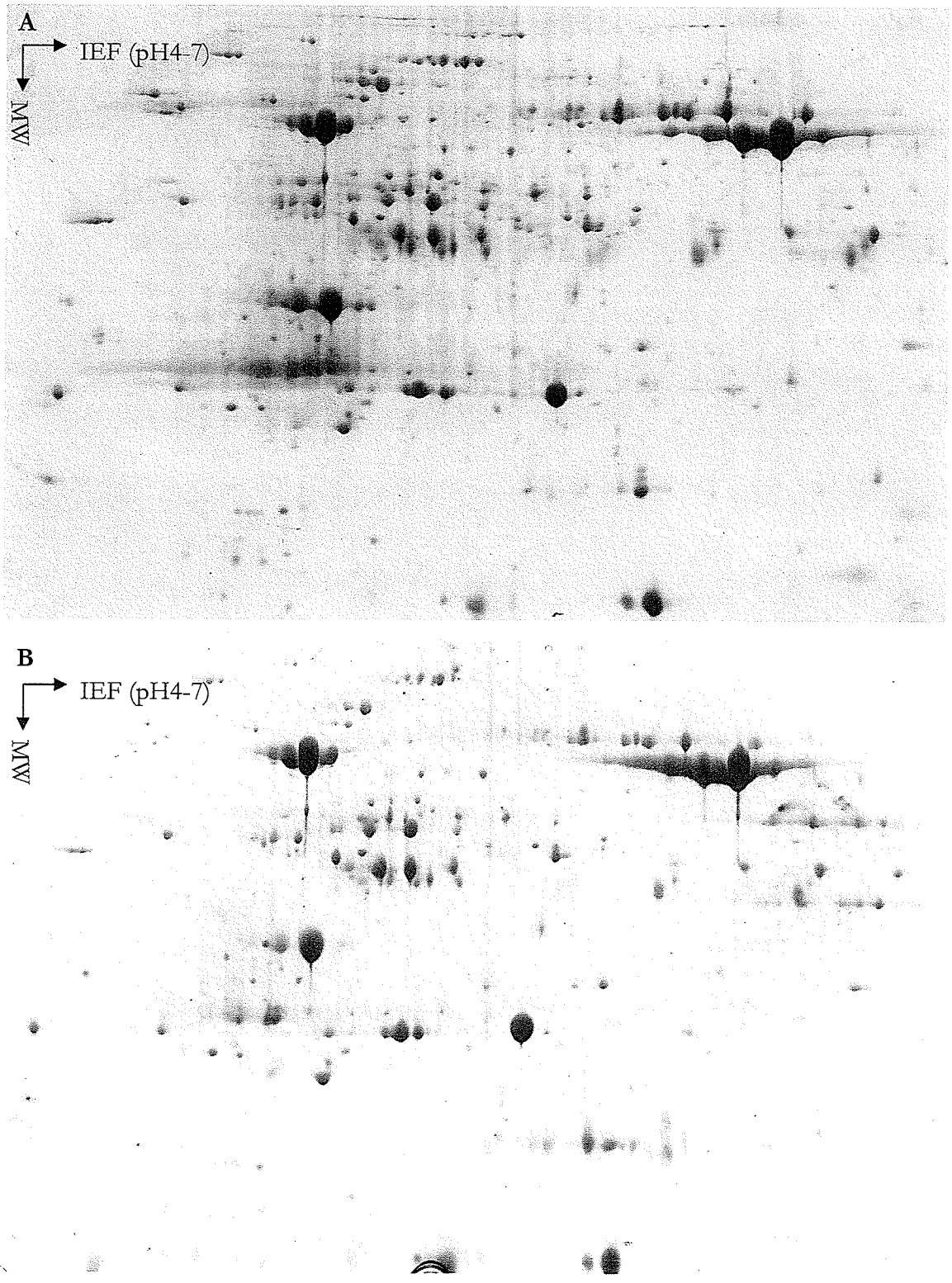
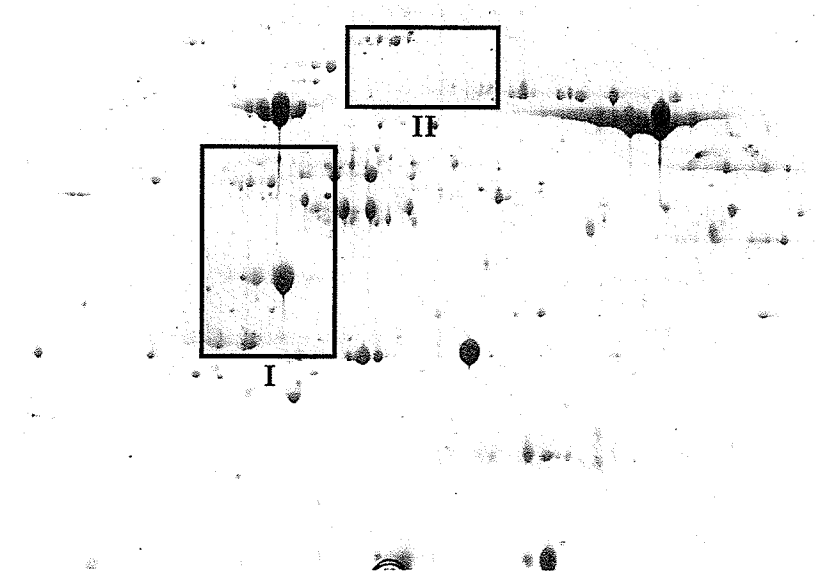
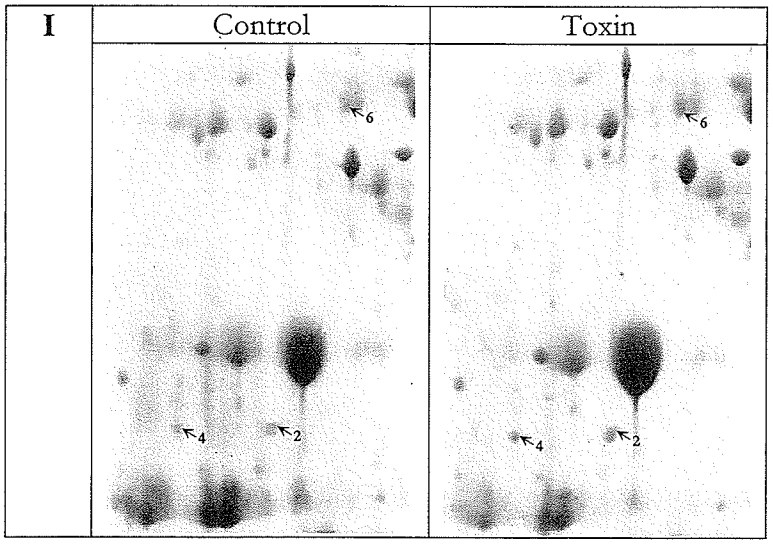


Figure 23. 2-DE separation of total soluble protein from Glenlea chloroplast inoculated with Ptr ToxA. Isolated intact chloroplasts were inoculated with 1ng of Ptr ToxA for every 400,000 chloroplasts in solution, for 1 hr. Proteins extracted were resolved on pH 4-7 strips in the first dimension and separated on 12% SDS PAGE in the second dimension and stained with Coomassie brilliant blue.





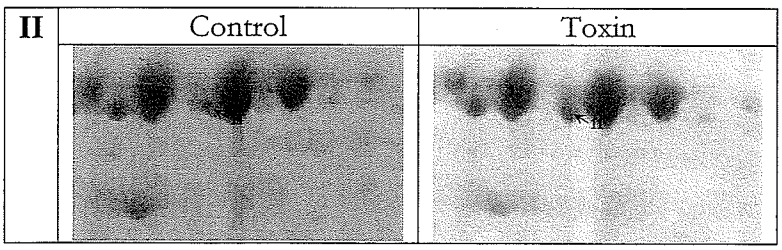


Figure 24. Protein Changes in 2D electrophoresis of Glenlea Chloroplast incubated with Ptr ToxA and control buffer. Regions of protein changes depicted in the representative chloroplast 2D gel were magnified in tables below the gel. The expression of spot 2, spot 4, and spot 6 (I) increased in chloroplast treated with toxin in comparison to the control. Spot 11 (II) showed an obvious increase in expression when treated with toxin.

Table 4: Identification of upregulated proteins in Glenlea chloroplast incubated with Ptr ToxA using MASCOT identification with MS/MS spectra

Spot # [a]	Putative Identification	Identification	Taxonomy	MS/MS MASCOT [b]	Score [c]	Percent Coverage
2	Hairpin Binding Protein 1	gi 38679331	*Triticum aestivum*	**K.INFDK** **R.GDRGELR** **K.DLDKLQGR** **K.LLSAVAGLNR** **K.FEITGIASIK** **R.LVYSSAFSSR** **R.GLAASQEDLDR** **R.TLGGSRPGPPTGR** **R.LLPITLGQVFQR** **R.QLEAAAPAPVDLAK** **K.GNLSQLPLLEVPR** **K.TKGNLSQLPLLEVPR** **R.GLAASQEDLDRADAAAR** **R.AVAAPSSAVDYSDTAAGAGDVPSLK** **R.IPDSLRPTTSNTGSGEFDVTYLDDDTR** **TOTAL**	29 27 46 35 65 67 75 28 79 110 121 119 46 65 60 972	63%
4	Hairpin Binding Protein 1	gi 38679333	*Triticum aestivum*	**K.DLDKLQGR** **K.LLSAVAGLNR** **K.FEITGIASIK** **R.LVYSSAFSSR** **R.GLAASQEDLDR** **R.TLGGSRPGPPTGR** **R.LLPITLGQVFQR** **R.QLEAAAPAPVDLAK** **K.TNGNLSQLPLLEVPR** **R.GLAASQEDLDRADAAAR** **R.IPDSLRPPASNTGSGEFDVTYLDDDTR** **TOTAL**	33 35 63 69 92 32 86 101 93 53 119 776	52%
6	ATP synthase CF1 beta chain	gi 14017579	*Triticum aestivum*	**R.SAPAFIELDTK** **R.AVAMSATDGLMR**	45 67	38%

				R.EGNDLYMEMK	26	
				K.AHGGVSVFGGVGER	65	
				R.IVGNEHYETAQR	22	
				R.FVQAGSEVSALLGR	75	
				R.VGLTALTMAEYFR	73	
				K.VALVYGQMNEPPGAR	81	
				R.TNPTTSPPGASTIEEK	63	
				TOTAL	517	
11	chloroplastic aldolase	gi 218155	*Oryza sativa*	K.TIASPGR	25	37%
				K.EGMFVK	9	
				R.TFEVAQK	49	
				K.EAAWGLAR	23	
				K.ANSLAQLGK	34	
				R.ALQNTCLK	25	
				R.AGAYDDELVK	58	
				R.AKANSLAQLGK	43	
				K.YTSDGEAAEAK	74	
				R.EAAYYQQGAR	84	
				R.LASIGLENTEANR	106	
				R.GILAMDESNATCGK	81	
				R.TVVSIPNGPSELAVK	83	
				K.RLASIGLENTEANR	76	
				R.GILAMDESNATCGKR	43	
				TOTAL	813	

(a) Spot numbers correspond to number in Figure # (Chloroplast zoomed spots)
(b) Matched peptide sequences in identified proteins by MASCOT MS/MS ion search
(c) MASCOT Ions score are -10*Log(P), where P is the probability that the observed match is a random event. Individual ions scores > 47 indicate identity or extensive homology ($p<0.05$).

Chapter 4: Discussion

4.1 Overview

Molecular events occurring between Ptr ToxA and its host are largely unknown, so a global approach was used to elucidate some of these events. Identification of upregulated and down regulated proteins using proteomics was key in giving insight into general molecular events occurring in infiltrated wheat leaves of sensitive and insensitive cultivars as a result of Ptr ToxA interactions. In the sensitive cutivar, Glenlea, eight proteins were identified, and in Amazon, seven were identified that underwent specific and reproducible changes. Thus the difference between these lines is not simply based on the ability to internalize Ptr ToxA as its differentiating factor but also based on other biochemical reactions.

4.2 Molecular Events in Sensitive Cultivars

4.2.1 Non-host Specific Defense Proteins

The proteins identified in Glenlea support one convergent conclusion derived from two sets of related proteins. One set is comprised of inducing factors that lead to ethylene synthesis and the other set is leading to the breakdown of nitrogen metabolism, which affects plant nutrition. Not all the proteins found were metabolic. Two stress related proteins were also induced. The more interesting of the two is GTP binding protein typA, spot 8, a protein that is part of the BipA family of ribosome binding protein family, produced in both plants and bacteria and involved in environmental and virulence related functions in bacteria (Farris *et al.*, 1998; Grant *et al.*, 2003). Typ A is

required by *Sinorhizobium meliloti* to establish nitrogen fixing symbiosis in *Medicago truncatula* (Kiss *et al.*, 2004). Although the exact role of typA protein in plants is unknown, such proteins have been seen to be downregulated in transcriptomic studies involving plant Avr-R gene interactions (Vergne *et al.*, 2007). This evidence suggests that typA, whether expressed in plants or bacteria, promotes symbiosis. The downregulation of this protein is observed in the Glenlea Ptr ToxA infiltrated leaf. The susceptible plant may have derived the ability to downregulate the gene as preventative defense against symbiosis of the pathogen.

Lignification was another defense mechanism activated, as seen with the sudden expression of cinnamyl alcohol dehydrogenase, spot 53, an enzyme that catalyses the production of cinnamyl alcohols, the precursors of lignin, from their corresponding cinnimaldehydes (Mitchell *et al.*, 1994) and is a molecular marker for lignin synthesis (Walter *et al.*, 1988). Lignification is one of the processes observed in plants in response to infection or pathogen challenge (Vance *et al.*, 1980) and functions primarily in building a physical barrier to limit further pathogen invasion (Ride *et al.*, 1975). In wheat, the inoculation of wounds with non-pathogenic fungi resulted in lignification at the site. Lignification is a defense mechanism of a non-host resistance in wheat. This observation suggests that preventative action, albeit non-specific, is being activated within plants.

4.2.2 Disease Propagation Signals

Interestingly, spot 34, identified to be Putative Ado Met synthase 3 or Adenosyl methionine synthase 3 showed a progressive increase thoughout day one to day three in Glenlea. It functions by converting methionine to Ado Met 3. In Arabidopsis a non

functional mutant of Ado Met 3 resulted in a significant reduction in lignin content (Shen *et al.*, 2002). An upregulation of S-adenosyl-methionine (Ado Met) synthase has been observed in lignifying tissue in tomatoes (Sanchez-Aguayo *et al.*, 2004), therefore Ado Met is required for lignification.

An increase in the production of Ado Met requires the presence of its precursor methionine, thus is it of interest to note that methionine synthase 2, spot 21, was only produced in the toxin infiltrated Glenlea leaves and increased progressively throughout day 1 to 3. Methionine is synthesized *de novo* by plants after inorganic sulfate assimilation and cysteine or homocysteine synthesis (Thomas and Surdin-Kerjan, 1997). Methinone synthase catalyses the last step of this process by transferring a methyl group to homocysteine.

Fungi, such as *Puccinia indica*, have also been shown to influence the methyl cycle through the increased synthesis of methionine synthase (Sarosh *et al.*, 2005) Once methionine is synthesized it can take one of two paths in the plant. It is either incorporated into proteins or converted into Ado Met. The increase of methionine synthase in this pathogen interaction is used for the synthesis of Ado Met which is a key player in many regulatory pathways. In this experiment we see an upregulation in both, thus, speculation is not required. Ado Met is increased, as demonstrated by the upregulation of adenosyl methionine synthase, Spot 34.

Ado Met is a substrate for a number of catalytic reactions and is a universal methyl donor for many biological methylations and acts as a precursor of, nicotinamines and phytosiderophores, polyamines and ethylene (Roje, 2006). The nicotiamides and phytosiderophores are required to bind metal ions (Takahashi *et al.*, 2003; Mizuno *et al.*,

2003) and mutational studies using plants with abnormal polyamine metabolism have suggested that these molecules are involved in the regulation of plant development (Hanzawa *et al.*, 2000, Imai *et al.*, 2004). Ethylene is also involved in plant growth and development, but more interestingly, is involved in responses to stress and pathogen interactions (Bleecker and Kende, 2000). Of these end products, only ethylene has been shown to be induced by pathogens. Thus the overproduction of Ado met is most likely to meet the need of ethylene production. The first commited step in ethylene biosynthesis is the conversion of Ado Met to 1-aminocyclopropane-1-carboxylic acid (ACC) which is catalysed by ACC synthase. ACC is then oxidized by ACC oxidase to ethylene (Moeder *et al.*, 2002).

Ethylene is a signalling molecule that has been shown to orchestrate many aspects of plant growth, such as leaf and floral senescence during fruit ripening and exposure to chemical inducers, and is an important mediator of plant responses to biotic and abiotic stress (Yang *et al.*, 1984; Kende, 1993; Wang *et al.*, 2002). Ethylene is involved in induced program cell death and in developmental programmed cell death (He *et al.*, 1996; Young *et al.*, 1997). Cell death and ethylene production are two early steps in elicitor induced defense response (Qin and Lan, 2004). Ethylene has also been shown to contribute to symptom formation and promote cell death in the susceptible response to virulent pathogens (Nurnberger and Scheel, 2001), as is the case here. The mechanism by which ethylene causes cell death is unclear, although it has been shown to activate the generating enzyme complex for reactive oxygen species (Chae and Lee, 2001).

In the hypersensitive response two bursts of reactive oxygen species are induced, a phenomenom that has also been observed in leaves exposed to O_3 (Schraudner *et al.*,

1998). In these leaves, the second O_3 induced burst was correlated with the distribution and size of the lesion. The spread of the lesion requires ethylene as demonstrated by Moeder et al (2002) who observed that an inhibition of ethylene inhibited the second O_3 burst and tissue damage was reduced. Thus, in the presence of oxidative species ethylene is involved in lesion amplification.

ROS were reported to be produced in wheat leaves treated with Ptr ToxA (Cuiffetti, 2005 unpublished) indicating that ethylene may be directly involved in the activation of ROS. However, based on the observation stated above, an alternate theory is that ethylene is not the cause of ROS in the plant but rather the amplifier. In this scenario, the initial oxygen species would most likely be provided by chloroplasts since their disruption results in the release of active oxygen species. This is a likely mechanism since chloroplasts are the suspected site of action for Ptr ToxA.

Ptr ToxA necrosis causes lesions which increase progressively and at 4 days post infiltration necrosis was observed throughout most of the infiltrated region. The infiltrated region was removed however the remaining green leaf and stem, was left to grow. Interestingly, the next day, the remaining half of the leaf began to show signs of necrosis, suggesting that necrosis is caused by signaling mechanisms in the plant. This suggests that the methionine, Ado Met pathway is most likely upregulated to provide precursors for the synthesis of ethylene and the role of ethylene in the host is to propagate signals of oxygen species and induce spread of necrosis or cell death, rather than induce production of ROS. Although this may partially explain the gross spread of necrosis, it is likely that more factors are involved.

4.2.3 Senescence Signals

Further analysis suggested that other plant proteins induced by Ptr ToxA may also be contributing to protective mechanisms but necrosis is still observed. This was observed through a chain of events starting with the induction of ATP-dependant Clp protease ATP binding subunits, spot 28 and spot 29. The ATP binding subunit was found in two separate spots of the same molecular weight, which is possibly due to a post translational modification or the presence of two isoform. Both spots were induced by Ptr ToxA. Spot29 was upregulated in treated leaves and spot 28 was only expressed in the treated leaves, therefore there is a general increase in Clp as result of host Ptr ToxA interaction.

Clps are part of the Hsp 100 family which are ubiquitously distributed among organisms. They mainly function as regulators of energy dependant protein hydrolysis but also as molecular chaperones. The majority of these proteins in plants are found in the chloroplast (Zheng *et al.*, 2002), which is the binding organelle of Ptr ToxA. Incidentally, an upregulation of a protein within this family was observed and identified to be a heat shock protein Hsp101, spot 45. But there is a significant increase in Clp protease ATP binding subunits, suggesting that this protein alone does not account for the general increase and that other Clps are being activated. There are about 19 Clps that have been identified since 2002 (Zheng *et al.*, 2002) each with a unique role. Some are involved in general degradation and other directly involved in photosynthesis, thus predicting the exact Clp that are affected as a result is difficult without further investigation.

The only Clp identified to be upregulated is the Hsp 101 which belongs to the caseinolytic (Clp) family of proteins and is structurally similar to ATP-dependant ClpB of *E.coli* (Kaityar-Agarwal *et al.*, 2001). They have two essential ATP binding sites and are a part of an even larger class of chaperones known as AAA+ chaperone like ATPases, which are involved in operation, assembly and disassembly of the protein (Neuwald *et al.*,1999). The Hsp100/ClpB proteins were induced as a result of heat stress and are involved in dissolution of protein aggregates (Glover and Lindquist, 1998; Goloubinoff *et al.*, 1999), however Hsp 101 is a protein that has been shown to be specifically induced with heat and is not present constitutively like other members of its family. Its overexpression aids in recovery of the plant after exposure to heat stress (Hong and Vierling, 2001). Although treated leaves were not exposed to heat in this study, it does demonstrate that Hsp 101 was capable of being induced as a result of pathogenic toxin stress of Ptr ToxA. Beyond its thermotolerance, Hsp 101 also has a translational regulatory function for certain cellular mRNAs (Wells *et al.*,1998). More specifically, it interacts with the regulatory sequence of ferredoxin mRNA, facilitates its transport to the chloroplast, and acts as a trans-acting factor for this gene (Ling *et al.*, 2000). The presence of HSP 101 induces a protective mechanism for ferredoxin. Ferrodoxin plays an important role in plant metabolism by distributing reducing equivalents to various electron consuming pathways, such as carbon dioxide assimilation and nitrogen assimilation (Togenetti *et al.* 2006). However, without ferredoxin NADP oxidoreductase (FNR), ferredoxin cannot be utilized. A general decrease in FNR, spot 5 was observed in Ptr ToxA infiltrated plants, which might suggest the increase in ferredoxin is not being utilized.

FNR exists in two forms. Photosynthetic ferredoxin NADP oxidoreductase requires ferredoxin to act as an electron acceptor in the carbon dioxide assimilation pathway. Heterotrophic FNR reduced ferredoxin, the reduced form of ferredoxin, is required by the nitrogen assimilation pathway (Bowhser *et al.*, 1992). Thus the presence of different ferredoxin NADP oxidoreductase affects different processes. Ferredoxin NADP(H) oxido reductase (FNR) was observed to decrease throughout day 1 to day3, but it was not possible to determine from the sequence whether it was heterotrophic or photosynthetic. The type of ferredoxin present is required to interpret the effect of this disease.

It can be speculated that heterotrophic FNR is decreasing because changes in glutamine synthase isoform GS1b, spot 66, were observed, an enzyme involved in nitrogen assimilation in plants. If hFNR is decreased it will result in a decrease in the reduced form of ferredoxin and the lack of the reduced ferrodoxin prevents nitrogen assimilation. It is important to note that primary nitrogen metabolism is controlled by glutamine synthase 2(GS2) so a decrease of hFNR will result in a decrease of GS2 and the upregulated nitrogen protein is glutamine synthase 1 (GS1). This increase in GS1 further suggests GS2 is decreased because GS1 has been shown to compensate for the decrease in GS2 (Pageau *et al.*, 2006) therefore there is a decrease in nitrogen metabolism.

Interestingly, the size of a lesion that develops when fungi or bacteria infect plant can change depending on plant nutrition (Hoffland *et al.*, 2000; Long *et al.*, 2000; Menzies 1991) and a reduction in nitrogen metabolism often increases the susceptibility in plants (Pageau *et al.*, 2006). Thus, a decrease in nitrogen assimilation in conjunction with the possibility of ethylene production could contribute to the cause and the spread of

necrosis, thus the hypothesis is ethylene is being produced as a result of the increased production of methionine and Ado Met.

The increase in GS1 not only signified a decrease in nitrogen metabolism but also indicates senescence is occurring. GS1 is involved in nitrogen mobilization which is a requirement of senescent leaves, and its increase signifies the activation of senescence (Pageau *et al.*, 2006). Senescence is also activated by other stresses such as ozone (Miller *et al.*, 1999). Additionally, ethylene has also been associated with interconnecting pathways involved in senescence of stress response (Buchanan-Wollaston, 1997). Therefore, Ptr ToxA interaction with sensitive cultivars induces senescence.

This observation is consistent with transcriptome analysis that have demonstrated that transcripts of senescence enhanced genes were increase in leaves exposed to fungus and bacteria (Butt *et al.*, 1998). It appears that Glenlea perceives Ptr ToxA as a pathogen attack, as shown by the changes in TypA and cinnamyl alcohol dehydrogenase, however is not capable of activating signals to prevent necrotic symptoms. Instead it seems the interaction with Ptr ToxA results in the induction of a protein that facilitates death, through changes in physiological processes that are similar to natural senescence. These events have been summarized in a hypothetical scheme, Figure 25.

4.3 Molecular Events in Insensitive Cultivars

4.3.1 Initial Senescent Signaling

Changes were also observed in the insensitive cultivar, however different effects were seen and over half of the seven proteins identified were involved in photosynthetic

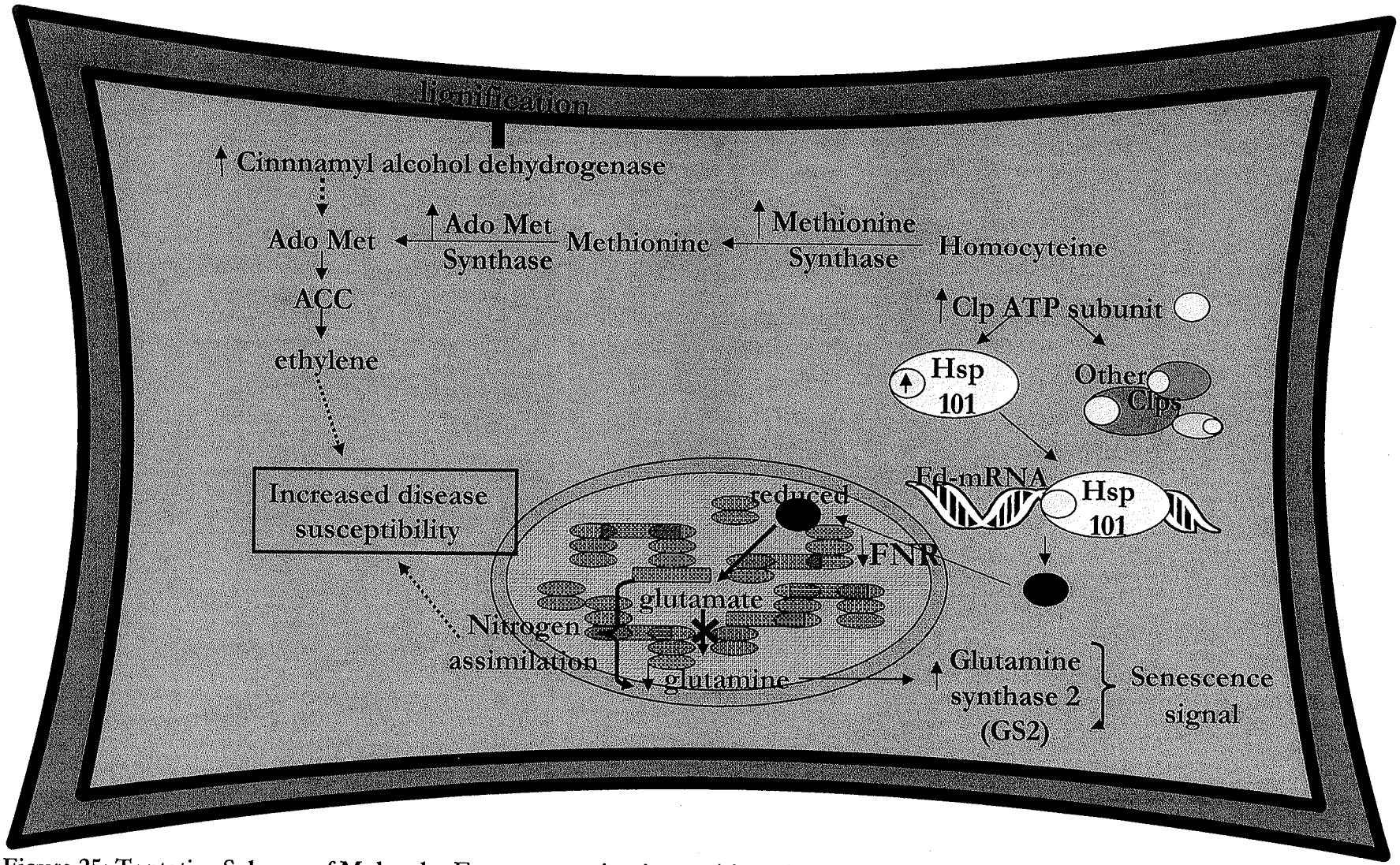


Figure 25: Tentative Scheme of Molecular Events occurring in sensitive wheat leaves as a result of Ptr ToxA interaction. Findings from the proteomics experiments support the involvement of senescence in the sensitive cultivar as seen by the increase in glutamine synthase 2. Disease susceptibility of Glenlea may be increased by the synthesis of ethylene precursor (AdoMet) and disruption of nitrogen metabolism. Proteins in **maroon** represent proteins identified in Table 2.

metabolism or photosynthesis. It appears as if the insensitive cultivar is also recognizing the presence of the toxin within the leaves because the pathway involved in mechanisms that aid in overcoming senescence are upregulated. Senescence can be seen as a form of programmed cell death since there is a signal to initiate it and a process required to complete it (van Doorn and Woltering, 2004). In mammalian cells, programmed cell death is a three step process; first there is activation of an inducing signal, then the transduction of a signal pathway, followed by a final common effector or execution pathway (Roberts *et al.*, 1999).

This has yet to seen in plants but it is possible that an inducing signal exists (Beers *et al*, 2000). One possible protein that may be acting as a signal is triticain beta, spot 1, a downregulation of this protein was identified in insensitive cell lines. Although articles on triticain have not been published, its role can be inferred by understanding its homologues. It is a cystiene proteinase with an N-terminus similar to some C1 peptidases, and shows similarity to papain (Abe *et al.*, 2006). An increase expression of papain cDNA homologues have been identified in several plant organs undergoing scenescence, as well as tissue undergoing programmed cell death (Beers *et al.*, 2000) and have been shown to be induced in instances of stress and plant pathogen interactions (Solomon *et al.*, 1999) and may be reduced to prevent initiation of the signal. However chloroplast were still seen to be affected in the insensitive wheat lines as seen by the decrease in LHCII CAB, spot 2. LHC II is associated mainly with the photosystem II (Sukenik *et. al.*, 1987) and the down regulation of the PSII promotes excess excitation energy that can leads to photoxidative damage (Karpinski *et. al.*, 2003).

Cystiene protease may be the inducing signal since one of the downstream proteins decreased in expression in Amazon leaves. During chloroplast development and in instance of stress, such as high light accumulation of serine/cystine proteases were implicated in their degradation of LHC II (Zelisko *et al.*, 2005). The same effect was observed in insentisitive leave infiltrated with Ptr ToxA. Perhaps a signal for senescence was not activated because the insensitive cultivar was able to downregulate the signal, despite this, a decrease in LHCII was still seen. However there was an upregulation of proteins that promote metabolic process within the chloroplast.

4.3.2 Inhibition of Senescence

During leaf senescence, chlorophyll and photosynthetic proteins are degraded (Humbeck *et al.*, 1996), and senescence can be accelerated by plant growth regulators such as ethylene (Smart, 1994) or slowed by inhibitors such as cytokinin. Inhibitors prolong the photosynthetic life span by inhibiting the degradation of chlorophyll and photosynthetic proteins (Bandenoch Jones *et al.*, 1996) by inducing synthesis and activity of hydroxypyruvate reductase (HPR) (Anderson *et al.*, 1996) and glyderaldehyde 3-phosphate dehydrogenase (Feierabend and de Boer, 1978). Both of these proteins were upregulated in insensitive leaves infiltrated with Ptr ToxA indicating that insensitve cultivars are actively inhibiting senescence.

HPR, spot 23 and 24, is an important enzyme associated in the recycling of carbons in the photorespiratory cycle (Murray *et al.*, 1989). It reduces hydroxypyruvate to D-glycerate (Stafford *et al.*, 1954) and a shortage of glycerate leads to a decrease in 3-phosphoglycerate which disrupts the Calvin cycle (Okinaka *et al.*, 2002). Therefore a

decrease in HPR activity leads to senescence and an increase in HPR prevents it. An increase in HPR is not possible if chloroplast are disrupted, thus the increase in HPR indicates intact healthy chloroplast are present in insensitive cultivars and contributes to the prevention of senescence. Furthermore, proteins involved in the Calvin cycle seem to be important in retarding the effects of senescent since disruption of the electron transport system in the light reaction could potential generate large amounts of free radicals that could lead to death (Okinaka *et al.*, 2002). Glyceraldehyde 3 phosphate dehydrogenase is another enzyme of the Calvin cycle that was upregulated in insensitive leaves as well as induced by cytokinin an inhibitor of senescence. Thus, taken together with the observation that sensitive leave are senescing, it appears that one of the mechanisms activated in insensitive plants is the prevention of necrosis through the activation of proteins that promote prolonged photosynthetic life span and retards senescence. Thus, in insensitive leaves senescence may be prevented by maintaining functional chloroplasts.

4.3.3 Protein Synthesis

Other proteins activated in the insensitive line were enzymes involve in protein synthesis. An increase in methylenetetrahydrofolate reductase, spot 4, an enzyme involved in the production of methionine was observed. This enzyme catalyzes the reduction of 5,10 methylenetetrahydrofolate to 5-methyltetrahydrofolate, which serves as a methyl donor for methionine synthesis from homocystine (Roje *et al.*, 1999) methionine is the first amino acid required for protein synthesis. An increase of elongation factor 1 gamma, spot 13, was also seen suggesting there is an increase in translocation resulting in

newly synthesized proteins. Re-evaluation of the unregulated proteins involved in the carbon cycles, spot 23-25, showed dramatic increases in each protein. This demand for newly synthesized proteins could easily account for the increase in protein production.

Elongation factor 1 is comprised of alpha, beta, beta', and gamma subunits (Hanbauer *et al.*, 2003) and an increase in elongation factor gamma contributes to the increase in elongation factor 1 but it also plays a specific role. A homologue of elongation factor gamma 1 has been demonstrated to be a component of a complex that binds to the promoter site of methionine sulfoxide reductase in yeast (Hanbauer *et al.*, 2003). Methionine sulfoxide reductase A reverses the oxidation of methionine residues which, in turn maintains the function of many proteins by preventing oxidative damage (Hanbauer *et al.*, 2003). Methionine is one of the major targets of reactive oxygen species (Moskovitz 2005), therefore the specific increase in elongation factor 1 gamma like protein suggests that active oxygen species are being produced in the cell and it's upregulation could indicate an induction of protective mechanisms.

4.3.4 Putative Chloroplast Transport Protein

Another chloroplast protein, Rieske Fe-S, spot 10 and 11 was also identified. Rieske Fe-S is a protein that is required by cytochrome b_6/f complex for structural stability (Kim *et al.*, 1998), and is essential for photosynthesis. *Chlamydomonas reinhardtii* mutants that lacked functional Rieske protein resulted in complete suppression of photosynthetic electronflow (Maiwald *et al.*, 2003). However this is just one of the functional roles of Rieske Fe-S in the chloroplast. It also uses Rieske Fe-S as a transport machinery. Rieske Fe-S is a substrate of the thylakoid Tat pathway. Protein transport by

this pathway is mediated by thylakoid targeting signals peptides (Chaddock *et al.*, 1995) and has the ability to translocate fully folded proteins across the thylakoid membrane (Clark and Theg, 1997). In Rieske Fe-S, the NH_2 terminal is a thylakoid- targeting domain and once targeted, the protein is anchored to the luminal side of the thylakoid by the NH_2 terminal domain (Madueno *et al.*, 1994) and is capable of translocation or insertion of proteins into the thylakoid membrane (Molik *et al.*, 2001).

It is interesting to speculate on the exact role of Rieske in this system. As mentioned previously, Reiske protein was identified in two spots, which suggests a possibility for post translation modification or the existence of an isoform. However only one form exists in each treatment, the Rieske protein with the higher pI is only seen in the control and the lower pI form is only seen in the toxin infiltrated leaves. Interestingly, the lower pI form seen in both the control and toxin infiltrated Glenlea, which means no change is occurring in Glenlea. The difference in protein isoforms in Amazon, suggest it is playing a role in transport and the two forms are most likely the result of a modifications after protein transport and the presence of the same form in Glenlea indicates the inability of Rieske Fe-S to act as a transporting factor in the sensitive wheat line. If true, then this may be the key differentiating factor between the chloroplast of the two cultivars. Perhaps Amazon is able to initiate or respond to inhibition of senescence because it is capable of importing a protein into the chloroplast that promotes its survival. These findings are summarized into a scheme in Figure. 26.

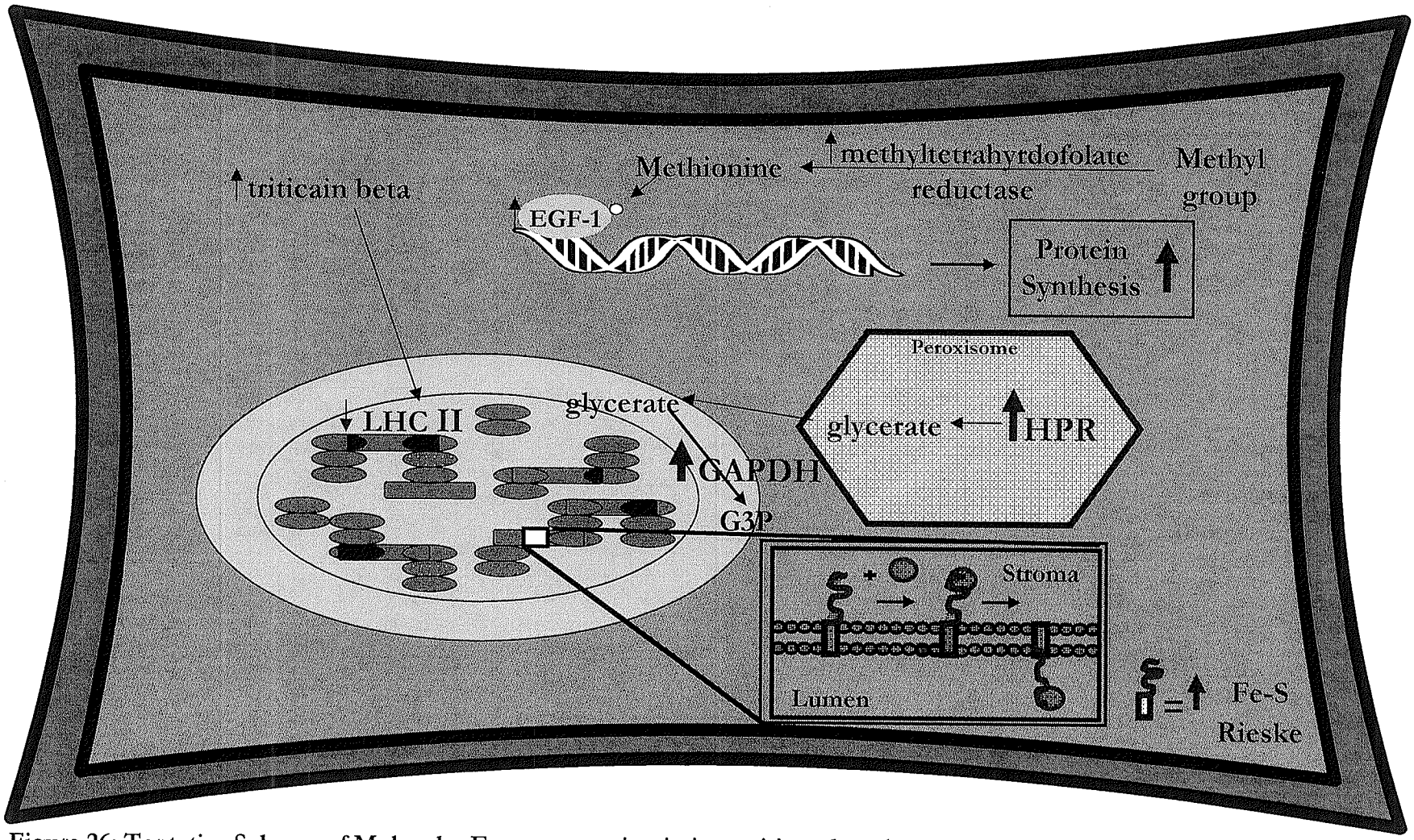


Figure 26: Tentative Scheme of Molecular Events occurring in insensitive wheat leaves as a result of Ptr ToxA interaction. A senescence signal, triticain beta, was shown to be increased in the insensitive cultivar however the over production of proteins involved in the inhibition of senescence (HPR and GAPDH) prevented senescence. The internalization of proteins by Fe-S Rieske may be an important differentiating factor between Glenlea and Amazon chloroplasts. HPR-hydroxypyruvate reductase, GAPDH-Glyceraldehyde 3 phosphate dehydrogensase, G3P-Glyceraldehyde 3 Phosphate, LHCII-Light harvesting complex II. Protein in **purple** are identified in Table 3.

4.4 Initiation of Protective Mechanisms by Chloroplast

Chloroplasts play an important role in propagating necrosis but also an important role in preventing senescence signaling and the role it plays is dependant on the cultivar signaling mechanisms. In senescence, both the structure and the role of chloroplast are altered (Gover and Mohanty 1993), therefore the chloroplast proteome was examined to see if more proteins involved in Ptr ToxA interaction could be identified. One of the disadvantages of proteomic analysis through 2DE is that low abundance proteins are hard to visualize as it is limited by sensitivity of Coomassie blue stain and they may be masked by other proteins of higher abundance. Examining the chloroplast directly partly overcomes these disadvantages. An isolated population of chloroplast treated with toxin at a concentration that simulated the treatments of infiltrated leaves were compared for protein changes. A further advantage of working with an isolated chloroplast population is the ability to achieve a synchronous response. They were treated with the toxin for one hour because activated signaling mechanisms such as phosphorylation should have immediate effects, thus the proteome along with the phosphoproteome of the chloroplast was examined. However no reproducible changes were observed in the phosphoproteome, and those that did change localized to spots with protein concentration that were not sufficient for identification by tandem mass spectrometry.

A few changes were observed in the Coomassie stained proteome of the chloroplast. Reproducible changes were not seen in chloroplasts isolated from Amazon, the insensitive line. It is interesting that Amazon does respond to the toxin, since previous published data (Manning, 2006) had indicated that both cell lines had the capability to respond to the toxin and that the limiting factor was the lack of

internalization in insensitive cell line. However four spots did exhibit consistent changes within the two replicates of Glenlea chloroplasts incubated with toxin. A total of three proteins were identified all of which were seen to be upregulated in response to the toxin. Spot 2 and 4 was a hairpin binding protein 1, spot 11 was chloroplastic aldolase, and spot 6 was ATP synthase. The upregulation of a hairpin binding proteins is most likely the result of activation of general host defenses since hairpin structure are a feature used by viruses for minus strand RNA synthesis (Haasnoot *et al.*, 2000). The upregulation of this binding protein most likely aids in the prevention of viral replication, indicating pathogen stress is being recognized, albeit non-specifically since chloroplast have been incubated with a toxin and not a virus.

The perception of stress was also seen by the upregulation of chloroplastic aldolase, which is involved in the sugar phosphate pathway of green chloroplasts. It catalyzes the formation of fructose 1, 6-bisphosphate from dihydroxyacetone 3-phosphate and glyceraldehydes 3 phosphate(Michelis and Gepstein, 2000). Chloroplast aldolases are induced by heat, suggesting that this may be a common mechanism of protection for chloroplasts under stress conditions (Michelis and Gepstein, 2000). A protective role was also identified in the other protein ATP synthase CF1 beta. An increase expression of ATP synthase as well as CF_1-β unit occurred in instances of high light stress (Chow and Anderson, 1987). The activation of ATP synthase decreases the proton gradient across the thylakoid membrane and enhance energy transduction between PSII and PSI (Braun *et al.*, 1991). Jiao *et al.*, 2004, has suggested that this increase may indicate a regulatory pathway that prevents over protonation of the

thylakoid lumen as well as photooxidative damage of the photosynthetic apparatus as a result of light stress.

The proteins activated by Glenlea chloroplast treated with Ptr ToxA are inducible by stress. Since chloroplasts were only incubated for one hour, these proteins represent an initial response, which implies that recognition of the toxin by the chloroplast is occurring but does not result in immediate signals for senescence but rather an upregulation of general defense proteins to various stresses. However these mechanisms are not sufficient in preventing symptoms, since necrosis is still observed. Senescence signals prevail in Glenlea, the sensitive cultivar, resulting in necrosis, while in Amazon the insensitive cultivar, senescence is inhibited through the induction of proteins that promote photosynthetic life span.

Chapter 5: Conclusion

Proteomic analysis of specific protein variations in insensitive and sensitive wheat cultivars resulted in the identification of eighteen proteins. Through functional analysis, signalling pathways were identified and identified changes of a protein were often supported by changes in a downstream protein. The experiment demonstrated that proteomics is a very useful tool for the analysis of plant pathogen interactions involving toxins, such as Ptr ToxA.

The propagation of senescent signals within sensitive cultivars triggered by the Ptr ToxA leads to necrosis of the wheat leaves. Senescence signals prevailed over the upregulation of general defence proteins in the leaves, as well as the initiation of protective mechanisms upregulated by the chloroplast in response to the toxin. Insensitive cultivars were also capable of toxin recognition as indicated by the observed upregulation in senescence proteins; however a gross upregulation of proteins involved in the inhibition of senescence prevented necrotic symptoms. Therefore, necrosis induced by Ptr ToxA requires the activation of senescent proteins.

Chapter 6: Literature Cited

Abe,K., Kiyosaki,T., Matsumoto,I., Arai,S. and Asakura,T. 2006.Ocurrence of multiple cysteine proteinases showing different response to gibberellin in wheat seeds Unpublished (gi:111073717) http://www.ncbi.nlm.nih.gov

Allen, R.L. Bittner-Eddy, P.D., Grenville-Briggs, L.J., Meitz, J.C., Rehmany, A.P., Rose, L.E., and Beynon, J.L. 2004. Host-parasite coevolutionary conflict between *Arabidopsis* and downy mildew. Science 306: 1957-1960

Alvarez, S., Goodger, J.Q.D., Marsh, E.L., Chen, S., Asirvatham, V.S., Schachtham, D.P. 2006. J. Characterization of the maize xylem sap proteome. Proteome Research 5: 963-972

Anderson, B. R., Jin, G., Chen, R., Ertl, J. R., Chen, C. M. 1996. Transcriptional regulation of hydroxypyruvate reductase gene expression by cytokinin in etiolated pumpkin cotyledons. Planta 198: 1-5.

Badenoch-Jones, J., Parker, C. W., Letham, D. S., Singh, S. 1996. Effect of cytokinins supplied via the xylem at multiples of endogenous concentrations on transpiration and senescence in derooted seedlings of oat and wheat. Plant Cell Environ. 19: 504-516.

Ballance, G. M., Lamari, L. 1998. Molecular aspects of host-pathogen interactions in tan spot of wheat. Can. J. Plant Pathol. 20: 425-427

Ballance, G.M., Lamari, L., Bernier, C.C. 1989. Purification and characterization of a host-selective necrosis toxin from *Pyrenophora tritici-repentis*. Physiol. Mol. Plant Pathol. 35:203-213

Ballance, G.M., Lamari, L., Kowatsch, R., Bernier, C.C. 1996. Cloning, expression, and occurrence of the gene encoding the Ptr necrosis toxin from *Pyrenophora tritici-repentis*. Mol. Plant Path., http://www.bspp.org.uk/mppol/1996/1209ballance/

Beers, E. P., Woffenden, B. J., Zhoa, C. 2000. Plant proteolytic enzymes: possible roles during programmed cell death. Plant Mol. Biol. 44: 399-415.

Bleecker, A. B., Kende, H. 2000. Ethylene: a gaseous signal molecule in plants. Annu. Rev. Cell Dev. Biol. 16: 1-18.

Bowsher, C. G., Bouleton, E. L., Rose, J., Nayagam, S., Emes, M. J. 1992. Reductant for glumate synthase is generated by the oxidative pentise phosphate pathway in non photosynthetic root plastids. Plant J. 2: 893-898.

Braun, G., Evron, Y., Malkin, S., Avron, M. 1991. Proton flow through the ATP synthase in chloroplasts regulates the distribution of light energy between PSI and PSII. FEBS Lett. 280: 57-60.

Buchanan-Wollaston, V. 1997. The molecular biology of leaf senescence. J. Exp. Botany 48: 181-199.

Butt, A., Mousley, C., Morris, K., Beynon, J., Can, C., Holub, E., Greenberg, J. T., Buchanan-Wollaston, V. 1998. Differential expression of a senescence-enhanced metallothionein gene in *Arabidopsis* in response to isolates of *Peronospora parasitica* and *Pseudomonas syringae*. Plant J. 16: 209-221.

Carpentier, S.C., Witters, E., Laukens, K., Deckers, P., Swennen, R., Panis, B. 2005. Preparation of protein extracts from recalcitrant plant tissue: An evaluation of different methods for two dimensional gel eletrophoresis analysis. Proteomics 5: 2497-2507

Cary, L.A., Han, C.D., Guan, J.L. 1999. Intergrin-mediated signal transduction pathways. Histol. Histopathol. 14: 1001-1009

Chaddock, A. M., Mant, A., Karnauchov, I., Brinks, S., Herrmann, R. G., Klosgen, R. B. 1995. A new type of signal peptide: central role of a twin-arginine motif in transfer signals for the ΔpH-dependent thylakoidal protein translocase. EMBO J. 14: 2,715-2,722.

Chae, H. S., Lee, W. S., 2001 Ethylene- and enzyme-mediated superoxide production and cell death in carrot cells grown under carbon starvation. Plant Cell Rep. 20: 256-261.

Chen, S., and Dickman, M.B. 2004. Bcl-2 family member localized to tobacco chloroplast and inhibit programmed cell death induced by chloroplast-targetted herbicides. J. Exp. Bot. 55: 2617-2623

Chow, W. S., Anderson, J. M. 1987. Photosynthetic responses of *Pisum sativum* to an increase in irradiance during growth. II. Thylakoid membrane components. Aust. J. Plant Physiol. 14: 9-14.

Ciuffetti, L.M., Tuori, R.P. 1999. Advances in the characterization of the *Pyrenophora tritici-repenti* wheat interaction. Phytopathology 89: 444-449

Ciuffetti, L.M., Tuori, R.P. Gaventa, J.M. 1997. A single gene encodes a selective toxin causaul to the development of tan spot of wheat. Plant Cell 9: 135-144

Ciuffetti, L.M., Tuori, R.P., Gaventa, J.M. 1997. A single gene encodes a selective toxin casaul to the development of tan spot of wheat. Plant Cell 9:135-144

Clark, E.A., Brugge, J.S. 1995. Integrins and signal transduction pathways: the road taken. Science 268: 233-239

Clark, S. A., Theg, S. M. 1997. A folded protein can be transported across the chloroplast envelope and thylakoid membranes. Mol. Biol. Cell 8: 923-934.

Coppolino, M.G., Dedhar, S. 2000. Bi-directional signal transduction by integrin receptor. Int. J. Biochem. Cell Biol. 32: 171-188

da Luz, W.C., Hosford, R.M. 1980. Twelve *Pyrenophora trichostoma* races for virulence to wheat in the Central Plains of North America. Phytopathology 70: 1193-1196

Davletova, S., Rizhsky, L., Liang H., Shenggiang, Z., Oliver, D.J., Coutu, J., Shulaev, V., Schlauch, K., Mittler, R. 2005. Cytosolic ascorbate peroxidase 1 is a central component of the reactive oxygen gene network of *Arabidopsis*. Plant Cell 17: 268-281

Dhingra, O.D., Sinclair, J.B., 1985. Basic Plant Pathology Methods. CRC Press, Inc., Boca Raton, FL, 355pp.

Dodds, P.N., Lawrence, G.J. Catanzariti, A.M., Ayliffe, M.A., Ellis, J.G. 2004. The *Melampsora lini AvrL567* avirulence genes are expressed in haustoria and their products are recognized inside plant cells. Plant Cell 16: 755-768

Dushnicky, L.G., Balance, G.M., Sumner, M.J., MacGregor, A.W. 1996. Penetration and infection of susceptible and resistant wheat cultivars by a necrosis toxin-producing isolate of *Pyrenophora tritici-repentis*. Can J. Plant Pathol. 18: 392-402

Faik, A., Laboure A.M., Gulino, D., Mandaron, P., Falconet, D. 1998. A plant surface protein sharing structural properties with animal integrins. Eur. J. Biochem. 253: 552-559

Faris, J.D., Anderson, J.A., Francl, L.J., Jordahl, J.G. 1996. Chromosomal location for gene conditioning insensensitivity in wheat to a necrosis-inducing cultrure filtrate from *Pyrenophora tritici-repentis*. Pytophthology 86: 459-463

Farris, M., Grant, A., Richardson, T. B., and O'Connor, C. D. 1998. BipA: a tyrosine-phosphorylated GTPase that mediates interactions between enteropathogenic *Escherichia coli* (EPEC) and epithelial cells. Mol. Microbial. 28:265-279.

Feierabend, J., de Boer, J. 1978. Comparative analysis of the action of cytokinin and light on the formation of ribulose-biphosphate carboxylase and plastid biogenesis. Planta 142: 75-82.

Flor, H.H. 1956. The complementary genetic systems in flax and flax rust. Adv. Genet. 8: 29–54.

Foyer, C.H., Lelandalis, M., Kunert, K. 1994. Photo-oxidative stress in plants. Physiol. Plant 92: 696-717

Freeman, T., Rasmussen, J.B., Francl, L.J., Meinhardt, S.W. 1995 Wheat necrosis induced by *Pyrenophora tritici-repentis* toxin. In Proceedings Microscopy and Microanalysis, G.W. Bailey, M.H. Ellisman, R.H. Hennigar, and N.J. Zaluzec, eds (New York: Jones and Begell) pp. 990-991

Friesen, T.L., Rasmussen, J.B., Kwon, C.Y., Ali, S., Francl, L.J., Meinhardt, S.W. 2002. Reaction of Ptr ToxA-Insensitive Wheat Mutants to *Pyrenophora tritici-repentis* Race 1. Phytopathology 92: 38-42

Glover, J. R., Lindquist, S., 1998. Hsp104, Hsp70, and Hsp40: a novel chaperone system that rescues previously aggregated proteins. Cell 94: 73-82

Goloubinoff, P., Mogk, A., Ben Zvi, A.P., Tomoyasu, T., Bukau, B. 1999. Sequential mechanism of solubilization and refolding of stable protein aggrgates by a bichaperone network. Proc. Natl Acad. Sci.USA 96: 13732-13737

Grant, A. J., Farris, M., Alefounder, P., Williams, P.H., Woodward, M. J., and O' Connor, C. D. 2003. Co-ordination of pathogenicity island expression by the BipA GTPase in enteropathogenic *Escherichia coli* (EPEC). Mol. Microbiol. 48:507-521.

Gray, J., Janick- Buckner, D., Buckner, B., Close, P.S., Johal, G.S. 2002. Light-dependant death of maize *lls1* cells is mediated by mature chloroplast. Plant Physiol. 130: 1894-1907

Grover, A., Mohanty, P. 1993. Leaf senescence-induced alterations in structure and function of higher plant chloroplasts. In: Abrol YP et al. (eds) Photosynthesis, Photoreaction to Plant Productivity, pp 225-255. Mohan Primlani for Oxford & IBH publishing, New Delhi.

Haasnoot, P. C. J., Brederode F. T., Olsthoorn, R. C. L., Bol, J. F. 2000. A conserved hairpin structure in Alfamovirus and Bromovirus subgenomic promoters is required for efficient RNA synthesis in vitro. RNA 6: 708-716.

Haen, K.M., Lu, H., Friesen, T.L., Faris, J.D. 2004. Genomic Targetting and High-Resolution Mapping of the *Tsn1*Gene in Wheat. Crop Sci. 44: 951-962

Hagborg, W. A. F. A device for injecting solutions and suspensions into this leaves of plants. Canadian Journal of Botany. (1970) 48: 1135-1136

Hanbauer, I., Boja, E., Moskovitz, J. 2003. A homologue of elongation factor 1γ regulates methionine sulfoxide reductase A gene expression is *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA 100: 8199-8204.

Hanzawa, Y., Takahaski, T., Michael, A. J., Burtin, D., Long, D., Pineiro, M., Couplant, G., Komeda, Y. 2000. *ALCAULIS5*, an *Arabidopsis* gene required for stem elongation, encodes a spermine synthase. EMBO J. 19: 4248-4256.

He, C. J., Morgan, P. W., Drew, M. C. 1996. Transduction of an ethylene signal is required for cell death and lysis in the root cortex of maize during aerenchyma formation induced by hypoxia. Plant Physiol. 112: 463-472.

Hoffland, E., Jeger, M. G., Van Beusichem, M. L. 2000. Effect of nitrogen supply rate on disease resistance in tomato depends on the pathogen. Plant and Soil 218: 239-247.

Hong, S. W., Vierling, E. 2001. Hsp101 ia necessary for heat tolerance but dispensable for development and germination in the absence of stress. Plant J. 27: 25-35.

Humbeck, K., Quast, S., Krupinska, K. 1996. Functional and molecular changes in the photosynthetic apparatus during senescence of flag leaves from field-grown barley plants. Plant Cell Environ. 19: 337-344.

Hynes, R.O. 1992. Integrin versitility, modulation and signalling in the cell adhesion. Cell 69: 11-25

Imai, A., Matsuyama, T., Hanzawa, Y., Akiyama, T., Tamaoki, M., Saji, H., Shirano, Y., Kato, T., Hayashi, H., Shibata, D., Tabata, S., Komeda, Y., Takahashi, T. 2004. Spermidine synthase genes are essential for survival of *Arabidopsis*. Plant Physiol. 135: 1565-1573.

Isberg, R.R., Nhieu, T.V. 1994. Binding and internalization of microorganism by integrin receptor. Trends in Microbiol. 2: 10-14

Jamet, E., Canut, H., Boudart, G., Pont-Lezica, R.F. 2006 Cell wall protein: a new insight through proteomics. Plant Sci. 11: 33-39

Jelling, J. and Leech, R. The importance of quantitative anatomy in the interpretaion of whole leaf biochemistry in species of Triticum, Hordeum and Avena. The New Phytologist. (1982) 92, 39-48

Jia, Y., McAdams, S.A., Bryan, G.T., Hershey, H.P., Valent, B. 2000. Direct interaction of resistant gene and avirulence gene products confers rice blast resistance. EMBO J. 19: 4004-4014

Jiao, S., Hilaire, E., Guikema, J. A. 2004. Identification and differential accumulation of two isoforms of the CF1-β subunit under high light stress in *Brassica rapa*. Plant Physiol. Biochem. 42: 883-890.

Kaityar-Agarwal, S., Agarwal, M., Gallie, D.R., Grover, A. 2001. Search for the cellular functions of plant Hsp100/Clp family proteins. Crit. Rev. Plant Sci. 20: 277-295

Kariola, T., Brader, G., Li, J., and Palva, E.T. 2005 Chlorophyllase 1, a damage control enzyme, affects the balance between defense pathways in plants. The Plant Cell 17: 282-294

Kende, H. 1993. Ethylene biosynthesis. Annu. Rev. Plant Physiol. Plant Mol. Biol. 44: 283-307.

Kim, H., Xia, D., Yu, C. A., Xia, J. Z, Kachurin, A., M., Zhang, L., Yu, L., Deisenhofer, J. 1998. Inhibitor binding changes domain mobility in the iron-sulfur protein of the mitochondrial *bc*1 complex from bovine heart. Proc. Natl. Acad. Sci. USA 95: 8026-8033.

Kiss, E., Huguet, T., Poinsot, V., and Batut, J. 2004. The *typA* gene is required for stress adaptation as well as for symbiosis of *Sinorhizobium meliloti* 1021 with certain *Medicago truncatula* lines. MPMI 17: 235-244.

Kotchoni, S.O., Gachomo, E.W. 2006. The reactive oxygen species network pathways: an essential prerequisite for perception of pathogen attack and the acquired diesease resistance in plants. J. Biosci. 31: 389-404

Kwon, C.Y., Rasmussen, J.B., Meinhardt, S.W. 1998. Activity of Ptr ToxA from *Pyrenophora tritici-repentis* requires host metabolism. Physiol. Mol. Plant Pathol. 52: 201-212

Lamari, L., Bernier, C.C. 1989a. Evaluation of wheat lines and cultivars to tan spot *(Pyrenophora tritici repentis)* based on lesion type. Can. J. Plant Pathol. 11:49-56

Lamari, L., Bernier, C.C. 1989b. Toxin of *Pyrenophora tritici-repentis*: host-specififcity, significance in disease, and inheritance of host reaction. Phytopathology 79: 740-744

Lamari, L., Bernier, C.C. 1989c. Virulence of isolates of *Pyrenophora tritici repentis* on 11 wheat cultivar and cytology of the differential host reactions. Can J. Plant Pathol. 11: 284-290

Lamari, L., Bernier, C.C. 1991. Genetics of tan necrosis and extension chlorosis in tan spot of wheat caused by *Pyrenophora tritici-repentis*. Phytopathology 81: 1092-1095

Lamari, L., Sayoud, R., Boulif, M., Bernier, C.C. 1995. Identification of new race in *Pyrenophora tritici-repentis*: implication for the current pathotype classification system. Can. J. Plant Pathol. 17: 312-318

Lamari, L., Strelkov, S.E., Yahyaoui, A., Orabi, J., Smith, R.B. 2003. The identification of two new races of *Pyrenophora tritici-repentis* from the host center of diversity confirms a one-to-one relationship in tan spot of wheat. Phytopathology 93: 391-396

Ling, J., Wells, D. R., Tanguay, R. L., Dickey, L. F., Thompson, W. F., Gallie, D. R. 2000. Heat shock protein HSP101 binds to the *Fed-1* internal light regulatory element and mediates its high translational activity. Plant Cell 12: 1213-1227.

Long, D. H., Lee, F. N., TeBeest, D. O. 2000. Effect of nitrogen fertilization on disease progess of rice blast on susceptible and resistant cultivars. Plant Disease. 84: 403-409.

Loughman, R. and Deverell, B.J. 1986. Infection of resistant and susceptible cultivars of wheat by *Pyrenophora tritici-repentis*. Plant Pathol. 35: 443-450

Madueno, F., Bradshaw, S. A., Gray, J. 1994. The thylakoid-targeting domain of chloroplast Rieske iron-sulfur protein is located in the N-termininal hydrophobic region of the mature protein. J. Biol. Chem. 269: 17,458-17,463.

Maiwald, D., Dietzmann, A., Jahns, P., Pesaresi, P., Joliot, P., Joliot, A., Levin, J. Z., Salamini, F., Leister, D. 2003. Knock-out of the genes coding for the Rieske protein and the ATP-synthase δ-subunit of Arabidopsis. Effects on photosynthesis, thylakoid protein composition, and nuclear chloroplast gene expression. Plant Physiol. 133: 191-202.

Manning, V.A. Ciuffetti, L.M. 2005. Localization of Ptr ToxA Produced by *Pyrenophora tritici-repentis* reaveals protein import into wheat mesophyll cells. The Plant Cell. 17: 3203-3212

Manning, V.A., Andrie, R.M., Trippe, A.F., Ciuffetti, L.M. 2004. Ptr ToxA requires multiple motifs for complete activity. Mol. Plant Microbe Interact. 17: 491-501

Manning, V.A., Hardison, L.K., Ciufetti, L.M. 2007. Ptr ToxA interacts with a chloroplast-localized protein. MPMI 20: 168-177

Meinhardt, S.W., Cheng, W., Kwon, C.Y., Donohue, C.M., Rasmussen, J.B. 2002. Role of the arginyl-glycyl-aspartic motif in the action of Ptr ToxA produced by *Pyrenophora tritici-repentis*. Plant Physiol. 130: 1545-1551

Menzies, J.G., Ehret, D.L., Glass, A.D.M., Samuels, A.L. 1991 The influence of silicon on cytological interactions between Sphaerotheca fuliginea and Cucumis sativus. Physiol. Mol. Plant Pathol. 39: 403-414

Michelis, R., Gepstein, S. 2000. Identification and characterization of a heat-induced isoform of aldolase in oat chloroplast. Plant Mol. Biol. 44: 487-498.

Miller, J. D., Arteca, R. N., Pell, E. J. 1999. Senescence-associated geneexpression during ozone-induced leaf senescence in *Arabidopsis*. Plant Physiol. 120: 1015-1024.

Mitchell, H. J., Hall, J. L., Barber, M. S. 1994. Elicitor-Induced cinnamyl alcohol dehydrogenase activity in lignifying wheat (*Triticum aestivum* L.) leaves. Plant Physiol. 104: 551-556.

Mittler, R. 2002. Oxidative stress, antioxidants, and stress tolerance. Trends in Plant Sci. 7: 405-410

Mizuno, D., Higuchi, K., Sakamoto, T., Nakanishi, H., Mori, S., Nishizawa, N.K. 2003. Three nicotianmine synthase genes isolated from maize are differentially regulated by iron nutritional status. Plant Physiol. 132: 1989-1997

Moeder, W., Barry, C. S., Tauriainen, A. A., Betz, C., Tuomainen, J., Utriainen, M., Grierson, D., Sandermann, H., Langebartels, C., Kangasjarvi, J. 2002. Ethylene synthesis regulated by biphasic induction of 1-aminocyclopropane-1-carboxylic acid synthase and 1-aminocycloprpane-1-carboxylic acid oxidase genes is required for hydrogen peroxide accumulation and cell death in ozone-exposed tomato. Plant Physiol. 130: 1918-1926.

Molik, S., Karnauchov, I., Weidlich, C., Herrmann, R. G., Klosgen, R. B. 2001.0The Rieske Fe/S protein of the cytochrome b6/f-complex: missing link in the evolution of protein transport pathways in chloroplasts? J. Biol. Chem. 276: 42,761-42,766.

Moskovitz, J. 2005. Methionine sulfoxide reductases: ubiquitous enzymes involved in antioxidant defense, protein regulation, and prevention of aging-associated diseases. Biochimica et Biophysica Acta (BBA)-Proteins & Proteomics 1703: 213-219.

Murray, A. J. S., Blackwell, R. D., Lea, P. J. 1989. Metabolism of hydroxypyruvate in a mutant of barley lacking NADH-dependent hydroxypyruvate reductase, an important photorespiratory enzyme activity. Plant Physiol. 91: 395-400.

Neuwald, A. F., Aravind, L., Spouge, J. L., Koonin, E. V. 1999. AAA+: a class of chaperone-like ATPases associated with the assembly, operation, and disassembly of protein complexes. Genome Res. 9: 27-43.

Nurnberger, T., Scheel, D. 2001. Signal transmission in the plant immune response. Trends. Plant Sci. 6: 372-379.

Okinaka, Y., Ching-Hong, Y., Herman, E., Kinney, A., Keen, N. 2002. The P34 syringolide elicitor receptor interacts with a soyabean photorespiration enzyme, NADH-dependent hydroxypyruvate reductase. MPMI 12: 1213-1218.

Orolaza, N., Lamari, L., Ballance, G. 1995. Evidence for host specific chlorosis toxin from *Pyrenophora tritici-repentis,* the causal agent of tan spot of wheat. Phytopathology 85, 1282-1287

Pageau, K., Reisdorf-Cren, M., Morot-Gaudry, J. F., Masclaux-Daubresse, C. 2006. The two senescence-related markers, *GS1* (cytosolic glutamine synthetase) and *GDH* (glutamate dehydrogenase), involved in nitrogen mobilization, are differentially regulated during pathogen attack and by stress hormones and reactive oxygen species in *Nicotiana tabacum* L. leaves. J. Experimental Botany 57: 547-557.

Pyke, K.A. and Leech, R.M. 1987. The control of chloroplast number in wheat mesophyll. Planta 170:416-420

Qin, W. M., Lan, W. Z. 2004. Fungal elicitor-induced cell death in *Taxus chinensis* suspension cells is mediated by ethylene polyamines. Plant Sci. 166: 989-995.

Rampitsch, C., Srinivasan, M. 2006. The application of proteomics to plant biology: a review

Rasmussen, J.B., Kwon, C.Y., Meinhardt, S.W. 2004. Requirement of host signalling mechanisms for the action of Ptr ToxA in wheat. European Journal of Plant Pathology. 110: 333-335

Raymond, P.J., Bockus, W.W., Norman, B.L. 1985. Tan Spot of winter wheat: Procedures to determine host response. Phytopathology 75: 686-690

Rees, R.G., Platz, G.J. 1983. Effect of yellow spot on wheat. Comparison of epidemics at different stages of crop development. Aust. J. of Agric. Res. 34: 39-46

Rees, R.G., Platz, G.J. 1990. Sources of resistance to *Pyrenophora tritici-repentis* in bread wheats. Euphytica. 45: 59-69

Rees, R.G., Platz, G.J., Mayer, R.J. 1982 Yeild losses in wheat from yellow spot: comparison of estimates derived from single tillers and plots. Aust. J. Agric. Res. 33: 899-908

Ride, J. P. 1975. Lignification in wounded wheat leaves in response to fungi and its possible role in resistance. Physiol. Plant Pathol. 5: 125-134.

Roberts, L.R., Adejei, P.N., Gores, G.J. 1999. Cathepsins as effector proteases in hepatocyte apoptosis. Cell Biochem. Biophys. 30: 71-88

Roje, S. 2006. S-Adenosyl-L-methionine: Beyond the universal methyl group donor. Phytochemistry 67: 1686-1698.

Roje, S., Wang, H, McNeil, S., Raymond, R. K., Appling, D., Shachar-Hill, Y, Bonert, H. J., Hanson, A. D.1999. Isolation, characterization, and functional expression of cDNAs encoding NADH-dependent methylenetetrahydrofolate reductase from higher plants. J. Biol. Chem. 274: 36,089-36,096.

Rossignol, M., Peltier, J.B., Mock, H., Matros, A., Maldonado, A.M., Jorrin, J.V. 2006. Plant proteome analysis: A 2004-2006 update. Proteomics 6: 5529-5548

Samuilov, V.D., Lagunova, E.M., Kiselevsky, D.B., Dzyubinskaya, E.V., Makarova, Y.V., Gusev, M. V. 2003. Participation of chloroplast in plant apoptosis. Bioscience Reports 23: 103-117

Sanchez-Aguayo, I., Rodriguez-Galan, J. M., Garcia, R., Torreblanca, J., Pardo, J. M. 2004. Salt stress enhances xylem development and expression of S-adenosyl-L-methionine synthase in lignifying tissues of tomato plants. Planta. 220: 278-285.

Sarma, G.N., Manning, V.A., Ciuffetti, L.M., Karplus, P.A. 2005. Structure of Ptr ToxA: An RGD-containing host selective toxin from *Pyrenophora tritici-repentis*. The Plant Cell 17: 3190-3202

Sarosh, B. R., Sivaramakrishnan, S., Shetty, H. S. 2005. Elicitation of defense related enzymes and resistance by L-methionine in pearl millet against downy mildew disease caused by *Sclerospora graminicola*. Plant Physiol. and Biochem. 43: 808-815.

Schilder, A.M.C., Bergstrom, G.C. 1995. Seed transmission of *Pyrenophora tritici-repentis*, the causal fungus of tan spot of wheat. Eur. J. Plant Pathol. 101:81-91

Schraudner, M., Moeder, W., Wiese, C., van Camp, W., Inze, D., Langebartels, C., Sandermann, H. 1998. Ozone-induced oxidative burst in the ozone biomonitor plant, tobacco Bel W3. Plant J. 16: 235-245.

Shen, B., Li, C., Tarczynski, M. C. 2002. High free-methionine and decreased lignin content result from a mutation in the *Arabidopsis* S-adenosyl-L-methionine sythetase 3 gene. Plant J. 29: 371-380.

Singh, P.K., Mergoum, M., Ali, S., Adhikari, T.B., Elias, E.M., Anderson, J.A., Glover, K.D., Berzonsky, W.A. 2006. Evaluation of elite wheat germ plasm for resistance to tan spot. Plant Dis. 90: 1320-1325

Slaymaker, D.H., Keen, N.T. 2004. Syringolide elicitor-induced oxidative burst and protein phosphorylation in soybean cells, and tentative identification of two affected phosphoproteins. Plant Sci. 166: 387-396

Smart, C. M. 1994. Gene expression during leaf senescence. New Phytol. 126: 419-448.

Solomon, M., Belenghi, B., Delledonne, M., Menachem, E., Levine, A. 1999. The involvement of cysteine proteases and protease inhibitor genes in the regulation of programmed cell death in plants. Plant Cell 11: 431-443.

Stafford, H. A., Magaldi A., Vennesland, B. 1954. The enzymatic reduction of hydroxypyruvic acid to D-glyceric acid in higher plants. J. Biol. Chem. 207: 621-629.

Strelkov, S.E., Lamari, L. 2003. Host-parasite interactions in tan spot [*Pyrenophora tritici-repentis*] of wheat. Can. J. Plant Pathol. 25:339-349

Strelkov, S.E., Lamari, L., Ballance, G.M. 1998. Induced chlorophyll degradation by a chlorosis toxin from *Pyrenophora tritici-repentis*.Can. J. Plant Pathol. 20: 428-435

Sutton, J.C., Vyn, T.J., 1990. Crop sequences and tillage practises in relation to disease of winter wheat in Ontario. Can. J. Plant Pathol. 12: 358-368

Takahashi, M., Terada, Y., Nakai, I., Nakanishi, H., Yoshimura, E., Mori, S., Nishizawa, N. K. 2003. Role of nicotianamine in the intracellular delivery of metals and plant reproductive development. Plant Cell 15: 1263-1280.

Thomas, D., Surdin-Kerjan, Y., 1997. Metabolism of sulfur amino acids in Sacccharomyces cerevisiae.Microbiology and Molecular Biology Reviews. 61:503-532.

Togenetti, V.B., Palatnik, J.F., Fillat, M.F., Melzer, M., Hajirezaei, M., Valle, E.M., Carrillo, N. 2006. Functional Replacement of Ferredoxin by a Cyanobacterial Plavodoxin in Tabacco Confers Broad Range Stress Tolerance. Plant Cell 18: 2035-2050

Tomas, A., Feng, G.H., Reeck, G.R., Bockus, W.E., Leach, J.E. 1990. Purification of a cultivar specific toxin from *Pyrenophora tritici-repentis*, causal agent of tan spot of wheat. Mol Plant-Microbe Interact 3:221-224

Tuori, R.P., Wolpert, T.J., Ciuffetti, L.M. 1995. Purification of immunological characterization of toxic components from cultures of *Pyrenophora tritici-repentis*. Mol. Plant-Microbe Interact. 8: 41-48

Tuori, R.P., Wolpert, T.J., Ciuffetti, L.M. 2000. Heterologous expression of functional Ptr ToxA. Mol. Plant-Microbe Interact. 13: 456-464

Vance, C. P., Kirk, T. K., Sherwood, R. T. 1980. Lignification as a mechanism of disease resistance. Annu. Rev. Phytopathol. 18: 259-288.

van Doorn, W.G. 2004. Is petal senescence due to sugar starvation? Plant Physiology 134: 35-42

van Doorn, W.G., Woltering, E.J. 2004. Senescence and programmed cell death: substance or semantics? J. Exp. Bot. 55: 2147-2153

Vergne, E., Ballini, E., Marques, S., Sidi Mammar, B., Droc, G., Gaillard, S., DeRose, R., Tharreau, D., Notteghem, J. L., Lebrun, M. H., Morel, J. B. 2007. Early and specific gene expression triggered by rice resistance gene *Pi33* in response to infection by *A CE 1* avirulent blast fungus. New Phytologist. 174: 159-171.

Walter, M. H., Grima-Pettenati, J., Grand, C., Boudet, A. M., Lamb C. J. 1998. Cinnamyl-alcohol dehydrogenase, a molecular marker specific for lignin synthesis: cDNA cloning and mRNA induction by fungal elicitor. Proc. Natl. Acad. Sci. USA 85: 5546-5550.

Walton, J.D. 1996. Host-selective toxins: agents of compatibility. Plant Cell 8: 1723-1733

Wang, K. L. C., Li, H., Ecker, J. R. 2002. Ethylene biosynthesis and signaling networks. Plant Cell 14: S131-S151.

Watson, B.S., Asirvatham, V.S., Wang. L., Sumner., L.W. 2003 Mapping the proteome of barrel medic (*Medicago trucatula*). Plant Physiol. 131: 1104-1123

Wells, D. R., Tanguay, R. L., Le, H., Gaillie, D. R. 1998. HSP101 functions as a specific translational regulatory protein whose activity is regulated by nutrient status. Genes Dev. 12: 3236-3235.

Yaeno, T., Matsuda, O., Iba, K. 2004. Role of chloroplast trienoic fatty acids in plant disease defense responses. The Plant Journal 40: 931-941

Yang, S. F., Hoffman, N. E. 1984. Ethylene biosynthesis and its regulation in higher plants. Ann. Rev. Plant Physiol. 35: 155-189.

Young, T. E., Gallie, D. R., Demason, D. A. 1997. Ethylene-mediated programmed cell death during maize endosperm development of wild-type and *shrunken2* genotypes. Plant Physiol. 115: 737-751.

Zelisko, A., Garcia-Lorenzo, M., Jackowski, G., Jansson, S., Funk, C. 2005. AtFtsH6 is involved in the degradation of the light-harvesting complex II during high-light acclimation and senescence. PNAS 102: 13,699-13,704

Zhang, H., Francl, L.J., Jordahl, J.G., Meinhardt, S.W. 1997. Structural and physical properties of a necrosis- inducing toxin from *Pyrenophora tritici- repentis*. Phytopathology 87:154-160

Zheng, B., Halperin, T., Hruskova-Heidingsfeldova, H., Adam, Z., Clarke, A.K. 2002. Characterization of chloroplast Clp proteins in Arabidopsislocalization, tissue specificity and stress responses. Physiol. Plant. 114: 92-101