Comparative Redox Proteomics to investigate role of Nox mediated redox signaling in *Fusarium graminearum* pathogenesis

By

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Comparative Redox Proteomics to investigate role of Nox mediated redox signaling in *Fusarium graminearum* pathogenesis

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

of

MASTERS OF SCIENCE

Lovingly dedicated

To

Maa and Papa

tryambakam yajāmahe sugandhim pusṭi-vardhanam
urvārukamiva bandhanān mrtyor mukshīya māmrtāt

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Abstract

Fusarium graminearum causes Fusarium Head Blight, (one of) the most destructive cereal diseases in Canada. Yield loss, quality degradation and mycotoxin production make *Fusarium* a multifaceted threat. Regulated production of reactive oxygen species by Nox enzymes is indispensable for fungal pathogenesis. *F. graminearum* Nox mutant Δ*noxAB* produced equivalent mycotoxin but caused reduced virulence than wild-type. We hypothesized that Nox mediated redox signaling may participate in *F. graminearum* pathogenicity. Two-DE and gel-free biotin affinity chromatography, followed by LC-MS/MS analysis were employed for a comparative redox-proteomics analysis between wild-type and Δ*noxAB* to identify proteins oxidized by Nox activity. Total 35 proteins, 10 by 2-DE and 29 by gel-free system, were identified. 34% proteins participated in fungal metabolism, 20% in electron transfer reactions and 9% were anti-oxidant proteins. The findings suggested that Nox mediated thiol-disulfide exchange in proteins provide a switch for redox-dependent regulation of metabolic and developmental processes during induction of FHB.

Abbreviations

2-DE	Two-Dimensional Gel Electrophoresis
3-ADON	3-acetyldeoxynivalenol
15-ADON	15-acetyldeoxynivalenol
ACN	Acetonitrile
AMP	Adenosine monophosphate
APS	Ammonium persulphate
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CID	Collision induced dissociation
CMC	Carboxy methyl cellulose
DON	Deoxynivalenol
EDTA	Ethylene-dinitrilo-tetraacetic acid
ESI	Electrospray ionization
$FADH_2$	Reduced Flavin adenine dinucleotide
FDK	Fusarium-damaged kernels
FHB	Fusarium Head Blight
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factors
GSH	Glutathione
GTP	Guanosine triphosphate
ICBN	International Code of Botanical Nomenclature
IEF	Isoelectric Focussing
IPG	Immobilized pH gradient
kV	Kilovolt
LC-MS/MS	Liquid Chromatography-Mass Spectrometry
MAPK	Mitogen activated protein kinase
mBBr	Monobromobimane
MudPIT	Multidimensional protein identification technology
MW	Molecular weight
m/z	Mass to charge ratio
NADPH	Reduced Nicotinamide adenine dinucleotide phosphate
NADH	Reduced Nicotinamide adenine dinucleotide
NCBI	National Centre for Biotechnology
NIV	Nivalenol
PMSF	Phenylmethylsulfonyl fluoride
PPP	Pentose phosphate pathway
PTM	Post translational modification
RT	Retention time
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS- polyacrylamide gel electrophoresis
SOD	Superoxide dismutases
TCA	Trichloroacetic acid
TEMED	N,N,N,N'-tetra methylethylenediamine

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   It can be found in thesis at page number 14.

INTRODUCTION

Chapter 1: Introduction

Fusarium graminearum Schwabe (teleomorph: *Gibberella zeae* (Schwein.) Petch) is a major phytopathogen causing Fusarium Head Blight (FHB) disease in wheat. It poses a multifaceted threat because the disease results in quantitative and qualitative losses as well as mycotoxin contamination of the wheat grains. Highly destructive and decades long FHB epidemics made this pathogen a foremost field of study towards the end of 20th century. Trichothecene biosynthesis was identified as a virulence trait and confirmed by disrupting genes involved in this pathway (Proctor et al., 1995; Desjardins et al., 1996). *F. graminearum* genome sequence was publicly released in 2003 which encouraged functional genomics research of this pathogen (Guldener et al., 2006a; Xu et al., 2006). The development of an Affymetrix gene chip (Guldener et al., 2006b) enabled the global gene expression studies while targeted gene mutation studies helped to identify several mitogen-activated protein kinases (MAPKs) (*mgv1, map1, gpmk1*) involved in pathogenesis (Hou et al., 2002; Urban et al., 2003; Jenczmionka et al., 2003).

To date, very little is known about the signalling events and molecular mechanisms behind FHB infection especially at protein levels. Recently the first report on phosphoproteome of *F. graminearum* was published postulating the roles of phosphorylation events in activating tricothecene pathway (Rampitsch et al., 2010). Other studies suggesting the role of Nicotinamide adenine dinucleotide phosphate oxidases (Nox) enzymes in fungal virulence (Egan et al., 2007; Segmüller et al., 2008) have revealed putative links between redox signalling and pathogenicity. Nox enzymes produce reactive oxygen species (ROS) in a tightly regulated manner and contribute to increase in cellular oxidation levels. Activity of Nox enzymes leads to thiol oxidation

resulting in disulfide bonds and protein sulfenic acid moieties, which can be readily reduced by cellular redox systems mainly glutathione/glutathione reductase (GSH/GSR) or thioredoxin/thioredoxin reductase (Trx/TrxR) systems. This redox cycling of cysteine residues plays regulatory roles in protein function and signal transduction. As compared to mammalian systems, very little information on fungal redox regulation is available. Two isoforms of Nox enzymes Nox A and B have been reported indispensable for fungal pathogenicity; Nox A triggers the development of fruiting bodies (Lara-Ortiz et al., 2003) whereas NoxB activity is detected in ascospore germination (Malagnac et al., 2004). *F. graminearum* Δ*noxAB*, a double deletion mutant lacking both isoforms of Nox enzymes (Nox A and B), has been investigated for virulence and tricothecene production. Interestingly, Δ*noxAB* produces toxin at levels equivalent to a wild type (WT) strain but causes significantly reduced virulence (Dr R. Subramaniam, personal communication, September 2008). Functional analysis and pathology tests of Nox mutants have established that these enzymes play a critical role in inducing FHB, but no work has been done to investigate redox control on *F. graminearum* virulence. Consequences of Nox activity and effects of ROS at translational and post-translational levels, acting as signals for inducing pathogenicity are yet to be determined.

In view of this, it was hypothesized that redox signaling mediated by Nox A and B may participate in *F. graminearum* pathogenicity. *F. graminearum* is an excellent candidate for proteomics because it has a fully sequenced well-annotated genome and can be grown *in vitro*. The objectives of this study were to study the *F. graminearum* redox-disulfide proteome, identify redox signalling components, proteins and cysteine oxidation sites targeted by Nox activity, and to examine their roles. A cysteine-targeted redox

proteomics approach was used to obtain and investigate the *F. graminearum* redox proteome. Subsequent comparative analysis of wild-type and Δ*noxAB* redox proteomes enabled the identification of Nox target proteins from the global population of redox responsive proteins. In future, the elucidation of redox signalling events leading to induction of FHB disease would aid in discovering new virulence factors and understanding the infection mechanism at molecular levels.

LITERATURE REVIEW

Chapter 2: Literature Review

2.1 Fusarium Head Blight

Fusarium head blight (FHB) is an extremely devastating disease of wheat, barley and other small grain crops globally, predominantly in humid and semi-humid areas. Over the last several years, FHB has been the most important cereal disease of eastern Canada, the eastern Canadian prairies and midwest United States. It was first described in 1884 as "wheat scab" by Smith in England, but later in 1920 Atantoff coined the term "Fusarium blight" based on the discoloration of the infected plant (Stack 2003). Presently authors use both "scab" (mainly in the USA) and "blight" concurrently to describe the disease (Wong et al., 1992; Parry et al 1995). The damaged kernels (seeds) due to FHB are widely referred to as "Fusarium-damaged kernels" (FDK) (Gilbert and Tekauz 2000).

The FHB disease complex includes 18 *Fusarium* species isolated from naturally infected wheat or barley spikes; and found capable of infecting spikes when artificially inoculated, though with varying levels of virulence (Shaner 2003). *Fusarium graminearum* is the most common species in North America and considered the most virulent (Bai and Shaner 2004). High humidity, prolonged wet periods and temperature ranging from 25-30 °C during flowering stage provide the best conditions for *F. graminearum* to start an infection. These conditions are rampant in farmlands of the Red river valley and Southern Manitoba making them the most problematic areas in western Canada. Due to the low moisture level and cooler summer temperatures in the western prairies than that in eastern, Manitoba and south-eastern Saskatchewan experience the highest severity. FHB is less prevalent in central and western Saskatchewan, and Alberta.

2.1.1 Concern behind FHB

The 1990s witnessed numerous FHB epidemics all across North America. The total losses to the cereal grain industry reached above $ 1 billion over this period in Canada (Gilbert and Tekauz 2000). Every year from 1993 to 1998 several disease incidences, at varying intensities, caused estimated losses of $300 million for Manitoba and $200 million for Quebec and Ontario (Windels, 2000). In the United States, losses due to FHB for all crops were assessed to be $2.7 billion from 1998 to 2000 (Nganje et al., 2002). The disease is becoming a rising danger to the world's food supply because the outbreaks also occur in Asia, Europe, and South America. Yearly recurrence of FHB and the devastating outcomes have fueled research to investigate this disease to find the most effective management.

Concern behind studying FHB is augmented because of its multifaceted threat. On the one hand, this disease causes yield and quality losses due to floret sterility and formation of discolored, withered and light test-weight kernels. This degrades the market value and presents difficulties in export and processing of infected grain. On the other hand, the pathogen produces significant levels of mycotoxins; the most common is deoxynivalenol (DON), in infected grain which raises health concerns in human and livestock (McMullen et al., 1997; Peraica et al., 1999).

2.1.2 History of FHB in Canada

Within a decade of its first description in 1884 in England, FHB was found widely spread in North America. The earliest documented epidemic was in 1919, which caused a loss of over 2 million metric tons of wheat and extended into the Canadian

prairies in Manitoba and Saskatchewan (Stack 2003). The 1940, 1942 and 1945 were three epidemic years in eastern Canada and particularly Ontario (Sutton, 1982). In the late 1970s, quantitative studies of *Fusarium* toxins became a focal point (Mirocha et al., 1977). Subsequent outbreaks witnessed across eastern Canada in 1978, 1980 and 1982 and western Canada in 1985 (Couture 1982; Abramson et al., 1987) established FHB as a destructive crop disease and spurred the urgency to study the pathogen and find resistant varieties. In Manitoba, disease severity and incidence of FHB caught attention from 1991 onwards (Wong et al., 1992). A major reason for elevated disease incidence was introduction of semi-dwarf wheat varieties, which were resistant to lodging but more prone to FHB. Most of the damaged wheat was the cultivar Roblin; 21.9 % of the bread wheat acreage in Manitoba in 1991. In 1993, the highest infection levels were recorded in south-central Manitoba (Gilbert et al., 1995). Wet weather in 2008 again resulted in elevated FHB levels and approximately 50% of the wheat grown was degraded (Canadian Grain Commission report, 2009).

2.1.3 Symptoms of FHB

Blight symptoms develop within 3 days after infection if environmental conditions are favorable. Infected wheat heads ripen prematurely and appear bleached; hence the disease is called Fusarium head blight. Partially white and partially green heads are diagnostic indicators in wheat field (Figure 2.1A). The pathogen may also infect the stem below the head, causing brown discoloration of stem tissue. Salmon-orange spore and hyphal masses are visible on infected spikelet and glumes. The infected grains are shriveled, light weight and chalky white in appearance (Figure 2.1B).

Figure 2.1A: Diagnostic symptom of FHB: partial bleaching of wheat head

Pest & Crop Newsletter, Issue 9, 2009.

I II

Figure 2.1B I. Salmon-orange spores of F. graminearum visible on glumes of wheat.

II. Healthy kernels (left) and Fusarium damaged kernels (right). Chalky white to pink discoloration of infected kernels is observed.

http://www.ag.ndsu.edu/pubs/plantsci/smgrains/pp804w.htm
NDSU Extension service, NDSU, Fargo. September 2008

2.1.4 Epidemiology of FHB

A disease triangle describes the components that are necessary to establish and develop a disease; and is composed of A) inoculum, B) host and C) favorable environmental conditions (Shaner, 2003). FHB has been shown to depend on the above three components of the disease triangle.

A). Inoculum

Ascospores are considered the primary inocula that initiate infection. Since FHB is a monocyclic disease, level of primary inoculum that is available to infect the host in the subsequent season is a deciding factor on epidemic severity and frequency (Sutton, 1982). Optimum temperatures for ascospore production range from 25 to 28°C. A drop in air temperature and rise in relative humidity triggers spore discharge while it is inhibited by intermittent rains. Studies have shown that FDK do not act as a source of inoculum (Gilbert and Fernando, 2004).

B). Host plant

F. graminearum has a wide host range (wheat, barley, oats and corn). The flowering and grain filling stages are considered the most vulnerable (Xu and Chen, 1993). In wheat, FHB infection occurs from anthesis to early grain development; and because this window is brief, infection is restricted to only one cycle per season. Disease incidence depends on host susceptibility/ resistance and the spread of pathogen post infection. Sumai 3 is a resistant wheat cultivar, and FHB symptoms usually do not spread from infected to uninfected spikelets (Bai and Shaner, 2004). Strange et al. (1974) identified twice the amount of choline content in susceptible spikes than resistant cultivar during anthesis. Higher content of chlorogenic acid was detected in susceptible cultivar

Nannong 824 than in Sumai 3 during flowering (Ye et al., 1990). Both these chemicals have been found to stimulate *Fusarium* growth in vitro.

C). Environmental factors

Relative humidity and temperature, during and after anthesis, determine the severity of FHB. The optimal temperatures for *F. graminearum* perithecial and ascospore production range from 15 to 28 ºC and 25 to 28 ºC respectively. Studies have shown that after dispersal, *G. zeae* ascospores germinated within 4-6 hrs at 20 ºC and a relative humidity of 92-94% (Beyer and Verreet 2005, Gilbert et al. 2008).

2.1.5 FHB control strategies

Best management of FHB requires an integration of multiple management strategies. Use of a single strategy is often futile when the environment is favorable for disease incidence. Growing resistant cultivars with a combination of practices like seed treatment, crop rotation and use of fungicides offer more promising results. A modern method is biological control which aims at disrupting the pathogen life cycle and/or encourages mycoparasitism to intervene in *Fusarium* infection process (Khan and Doohan et al. 2009).

2.1.5.1. Resistant cultivars

Schroeder and Christensen (1963) proposed two types of resistance in wheat: resistance to initial infection (refereed to as Type I resistance) and resistance to spread of blight symptoms within a spike (called as Type II resistance). They found that these two types of resistance varied independently among cultivars. Three other types of resistance to FHB have also been proposed, based on the ability to resist kernel infection (Type III), maintain the yield irrespective of the presence of the disease (Type IV) and degradation

or no accumulation of mycotoxins (Type V) (Mesterházy 1995; Miller et al. 1985; Wang and Miller 1988). Type I and II resistance are commonly used in breeding programs. Type II resistance has been extensively studied in wheat as it appears to be more stable and less affected by non genetic factors (Bai and Shaner 2004). To date Sumai 3, a Chinese wheat cultivar, and its derivatives represents the highest degree of type II resistance in wheat. Canada's first FHB-resistant wheat cultivar FT Wonder was released in 2002, which derives its resistance from the Brazilian cultivar 'Frontana' possessing Type 1 resistance. Despite great efforts, no completely resistant variety against *F. graminearum* is currently available. This is due to the complexity of resistance inheritance and poor agronomic characters linked to resistance in wheat crop.

2.1.5.2 Cultural control

Rotation of wheat with non-host crops reduces the abundance of inoculum in the field (Sutton, 1982; Parry et al., 1995). When corn preceded wheat, FHB incidence increased by 15% as compared to 4%, when wheat was preceded by alfalfa or oats (Pirgozliev et al., 2003). Schaafsma et al. (2005) reported that conventional tillage and no till systems contributed to severity of FHB epidemics. Planting cultivars which mature on different days or staggered planting of the crop is highly advised; this reduces the risk to the entire crop if FHB infection strikes.

2.1.5.3 Biological control

Biological control is an integral part of FHB management system and is compatible with other control strategies. Methods to disrupt fungal life cycle, treatment of crop residue to reduce the pathogen inoculum or application of *Fusarium* antagonists to wheat heads during anthesis are mostly useful. Ascospore production and dispersal at

pre-infection stage, and spikelet invasion and colonization post infection, are the potential targets for applying biological controls. This intervention may be achieved by several ways, primarily antibiosis, mycoparasitism, host competition, aborting or delaying the germination of the spores in the infection process, induced resistance and inhibition of mycotoxin synthesis (Khan and Doohan et al., 2009).

2.1.5.4 Chemical control: Use of fungicides

While employing chemical control in FHB management, the appropriate crop stage (timing) and an optimum rate of application are critical. Matthies and Buchenauer (2000) reported reductions in FHB severity by 50-80 % when fungicides were applied at early flowering stage of a wheat crop. Application of "Triazole fungicides" namely Tebuconazole, Metconazole or Prothioconazole is recommended, because they are systemic and have been shown to reduce both FHB and DON contamination (Klix et al., 2007). The chemical action of triazole based fungicides is to inhibit the 14α-demethylase, an enzyme that is essential for ergosterol biosynthesis.

2.2 *Fusarium graminearum* (teleomorph: *Gibberella zeae*)

The genus *Fusarium* includes several economically important fungal species that are plant-pathogenic and cause chronic and fatal toxicoses in humans and animals (Desjardins, 2006). *F. graminearum* was used to be considered as a single, panmictic species worldwide, but recent phylogenetic studies have suggested the existence of nine geographically structured and reproductively isolated species (or lineages) within the *F. graminearum* species complex (O' Donnell et al., 2004). Table 2.1 presents nine lineages with their geographical distribution. The name *F. graminearum* is restricted for the species most commonly associated with Fusarium head blight worldwide.

Table 2.1 Phylogenetically distinct lineages of *F.graminearum* species complex

Lineage	Taxon	Distribution
1	*F. austroamericanum*	South America
2	*F. meridionale*	South and Central America, South Africa, New Caledonia, Nepal and Korea
3	*F. boothii*	South Africa, Mesoamerica, Nepal, Guatemala and Korea
4	*F. mesoamericanum*	Central America and Pennsylvania
5	*F. acacia-mearnsii*	Australia and South Africa
6	*F. asiaticum*	Eastern China
7	*F.graminearum*	North America and most common worldwide
8	*F. cortaderiae*	South America and Oceania
9	*F. brasilicum*	South America and Oceania

2.2.1 Classification of *F. graminearum*

F. graminearum belongs to the phylum Ascomycota, class Ascomycetes, order Hypocreales, family Nectriaceae and genus *Fusarium*. It can reproduce both asexually (anamorph) and sexually (teleomorph). The sexual stage is classified as *Gibberella zeae*. Occurrence of morphologically distinct asexual and sexual forms, at different times or in different habitats, caused conflicts in fungal classification. A "dual nomenclature" system was stablished in the International Code of Botanical Nomenclature (ICBN) in 1910 (Hennebert, 1987). Teleomorphs have been conventionally classified and named differently from their anamorphs. *Gibberella zeae* is homothallic and capable of self-fertilization (O'Donnell et al., 2000).

2.2.2 Genome of *F. graminearum*

The complete genome sequence of *F. graminearum* was released in May 2003 by the Broad Institute at the Massachusetts Institute of Technology in Cambridge, Massachusetts, United States. The sequence is 36 Mb long, consisting of over 12,000 predicted protein-encoding genes and is anchored to a genetic map revealing four chromosomes. Seventeen percent of the predicted gene products show no significant homology to any known protein and are thus probably unique to this fungus (Cuomo et al. 2007).

2.2.3 Life cycle and infection process

Life cycle of *F. graminearum* is well studied (Guenther and Trail, 2005) (Figure 2.2). Understanding the developmental cycle of a pathogen is important because control methods are effective when they can disrupt a critical phase in this cycle. In spring, warm moist weather conditions favor the development and maturation of perithecia that

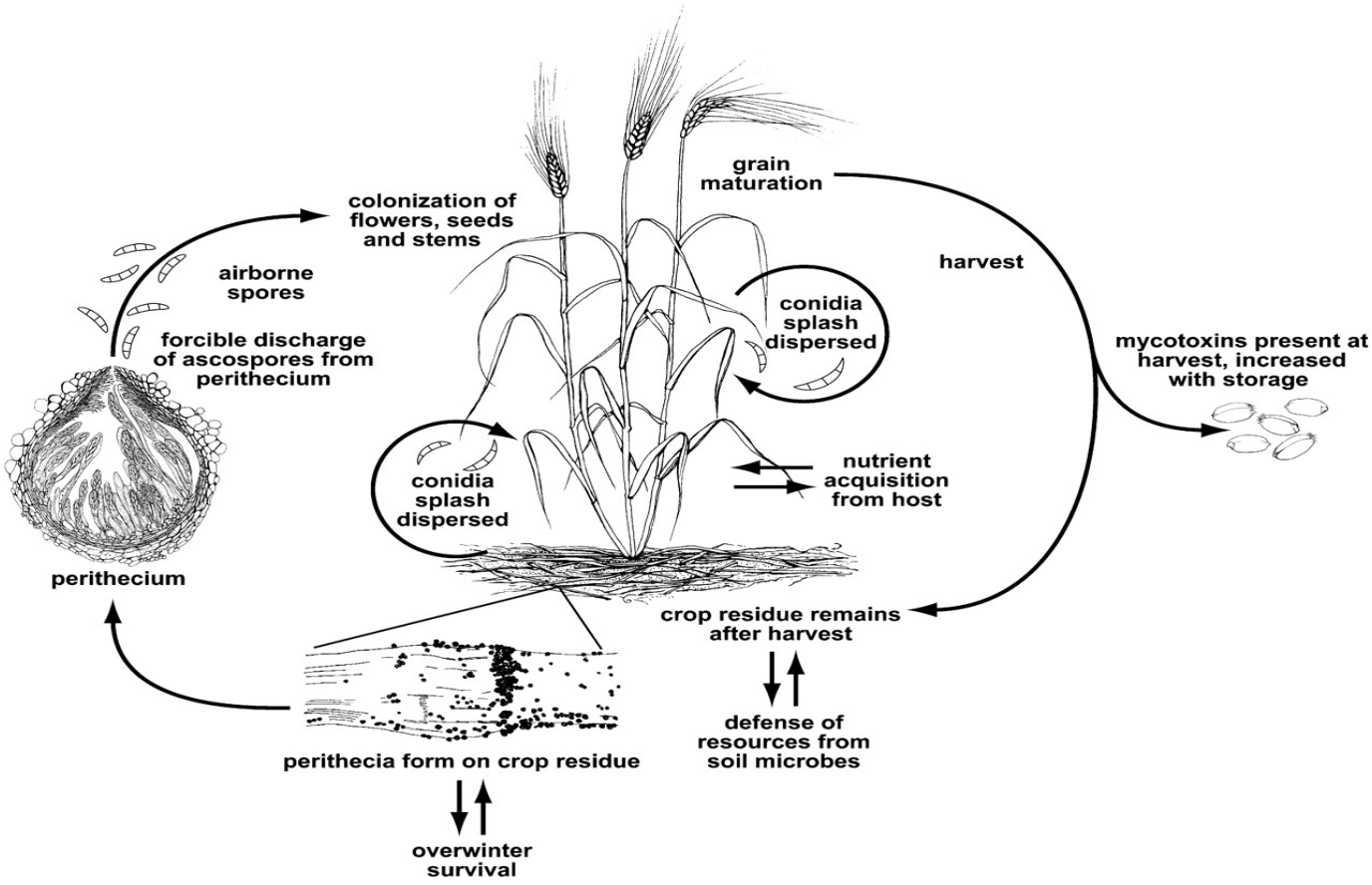


Figure 2.2 The life cycle of *Fusarium graminearum*

produce ascospores concurrently with the flowering of cereal crops. The sticky ascospores are forcibly discharged from mature perithecia and dispersed by wind, rain or insects to host plants. FHB is monocyclic; after one cycle of infection with ascospores, the fungus produces macroconidia by asexual reproduction. These structures overwinter saprophytically in crop debris and provide the source of primary inoculum for the next season. Post infection, the pathogen exhibits a brief asymptomatic period when it grows and colonizes the host; followed by necrosis of host tissues.

Unlike other filamentous fungi, *F. graminearum* doesn't form special infection structures (appressoria) for penetrating the host epidermis. It uses alternative ways to enter the host tissue, for example through the inner surfaces of florets, exposed anthers, open wounds or rarely through stomata (Bushnell et al., 2003). Anthers provide the most suitable route and act as an initial base for floret colonization (Kang and Buchenauer 2000a). It is believed that ascospores germinate within 6-12 hrs after landing (Pritsch et al., 2000). Post germination, hyphae travel inter- and intracellulary between the lemma and palea and proceed to developing kernels. The lemma and palea are two fibrous sheaths that protect the wheat seed. Once infected, florets fail to produce healthy grains. The hyphae spread within the entire head, successively destroying the fruit coat and finally the endosperm (Jansen et al 2005). *Fusarium* is reported to produce cellulases, xylanases and pectinases (Kang and Buchenauer 2000b); these enzymes may assist in its spread. The fungus colonizes the host through vascular tissue, blocking the phloem vessels. As a result the phloem ultimately collapses. This is the beginning of necrosis when thorough colonization by the fungus leads to plant death. Tricothecenes promote the spread of pathogen from an infected floret to adjacent ones. Jansen et al (2005)

demonstrated that spread of a non-DON producing *Fusarium* mutant was blocked by the development of heavy cell wall thickening in wheat, a host-defense response inhibited by the mycotoxin. Effective resistance to FHB requires strategies that conflict with the infection pathway. Detailed studies are required for targeting fungal metabolites or the detoxification of mycotoxins or using host defense genes known to be effective against other phytopathogens.

2.3 Trichothecenes

Fusarium species complex produces a number of chemically diverse toxins collectively called trichothecenes; the most common are deoxynivalenol (DON) and its 3-acetyl and 15-acetyl derivatives, nivalenol NIV, T-2 and HT-2 toxins. Chemically, trichothecenes are sesquiterpenoid mycotoxins, characterized by a tricyclic ring structure containing a double bond at C-9, 10 and an epoxide group at C-13. The toxicity results by interrupting peptidyl transferase enzyme activity on ribosomal protein L3 and inhibiting eukaryotic protein synthesis; (Desjardins, 2006).

Three strain-specific profiles of trichothecene biosynthesis called 'chemotypes' exist in the *Fusarium* species complex (Ward et al., 2002). These are NIV producers, 3ADON (DON producers that also make 3-acetyl deoxynivalenol) and 15ADON (DON producers that also make 15-acetyl deoxynivalenol). Chemotype diversity results from a loss of gene function; DON chemotypes contain a nonfunctional *tri13* pseudogene which prevents these strains from producing the C-4 oxygenated NIV (Kimura et al., 2003). Different chemotypes show varying levels of toxicities toward specific hosts. NIV-producers are more aggressive toward corn but less aggressive to wheat than DON producing strains (Carter et al., 2002; Cumagun et al., 2004).

2.3.1 Trichothecene biosynthesis

The trichothecene biosynthetic pathway has been detailed in *Fusarium sporotrichioides*. Comparative studies show that similar genes are functional in *F. graminearum*, with differences on acetylation (Kimura et al., 2003) or oxygenation reactions at positions C-4 and C-8 of the trichothecenes (McCormick et al., 2004). Trichothecene biosynthesis proceeds from farnesyl phosphate via trichodiene, followed by a sequence of sesquiterpene cyclization, eight oxygenations and four esterification steps (Desjardins, 2006). A total of fifteen genes have been identified in this pathway, found within either *F. sporotrichioides* or *F. graminearum* or in both species. Ten genes are suggested to form a gene cluster termed 'Tri-cluster', spanning a 23 Kb region of DNA in *F. sporotrichioides*.

2.3.2 'Tri cluster'

The 'Tri cluster' consists of central genes (*tri4* and *tri5*) and regulatory genes (*tri6* and *tri 10*). *tri4* and *tri5* are involved in early steps of trichothecene biosynthesis and are common to all chemotypes. The flanking genes (e.g. *tri3* and *tri13*) are involved in trichothecene structural pathways causing chemotype variations described above. *tri5* gene codes for trichodiene synthase, the enzyme catalyzing sesquiterpenoid cyclization and the first committed step in the trichothecene biosynthesis (Hohn and Beremand, 1989). *tri6* is a Cys_2His_2 zinc-finger protein involved in transcription activation of central genes as well as the flanking genes. *tri6* binds the promoter sequence TNAGGCCT of ten toxin biosynthetic genes, this binding is essential for transcriptional up-regulation. *tri10* acts upstream of *tri6* and required for expression of genes involved in toxin biosynthesis (Tag et al., 2001). Recently other genes outside the *tri*-cluster have also been found

involved. These include *tri1*, a cytochrome P-450 that catalyses oxygenation of trichothecene at positions C-7 and /or C-8; and *tri101* that is a trichothecene 3-O-acetyltransferase (McCormick et al., 2004; Kimura et al., 2003).

2.3.3 Deoxynivalenol (DON)

The most widespread mycotoxin produced by *F. graminearium* is deoxynivalenol (DON) (3α,7α)-3,7,15-trihydroxy-12,13-epoxytrichothec-9-en-8-one. It is a low molecular weight chemical (MW=296 Da) that has one primary and two secondary hydroxyl groups (Desjardins, 2006). DON toxicity symptoms in livestock include feed refusal, diarrhoea, emesis (hence also called as vomitoxin) and alimentary haemorrhaging. In humans, acute DON toxicity causes nausea, vomiting and loss of appetite; chronic exposure may lead to neurological disorders and immunosuppression (Bennett and Klich, 2003; Yazar and Omurtag, 2008).

DON phytotoxicity is an accumulative result of inhibition of protein synthesis, disruption of membrane integrity and alteration in electron transport and leaf chlorophyll content leading to wilting, chlorosis and necrosis in plants. Efforts have been made to develop trichothecene resistance; including alterations of the ribosomal target sites, inhibition of trichothecene biosynthesis and detoxification of trichothecenes (Desjardins, 2006). Some FHB-resistance cultivars are able to detoxify DON, primarily by glycosylation, acetylation or de-epoxydation reactions (Boutigny et al., 2008).

2.4. Virulence factors in *Fusarium* pathogenesis

Disruption studies of the *tri5* gene of *F. graminearium* resulted in the loss of DON production with significant reduction of virulence. This suggested that DON or

other trichothecenes might be the virulence factors in the plant-pathogen interactions (Proctor et al., 1995; Desjardins et al., 1996). Later investigations demonstrated that non-DON producing *Fusarium* strains can cause an initial infection without further colonization (Bai et al., 2002). Since then, DON has been designated as virulence enabler rather than a virulence factor. At the molecular level, pathogenesis is regulated by complex signalling events. Several mitogen activated protein kinases (MAPK) (Maf1, Mak2, Mgv1, Bmp1) and cAMP signalling regulate development of infection structures in many phytopathogenic fungi (Kojima et al., 2002; Solomon et al., 2005; Di Pietro et al., 2001; Zheng et al., 2000; Xu and Hamer 1996). López-Berges and group (2010) investigated the relationship between nitrogen signalling and the pathogenicity MAPK cascade in governing infection of the vascular wilt fungus *Fusarium oxysporum*. Nitrogen stress has been hypothesised to trigger virulence genes in fungal pathogens.

Nox enzymes were recently found to be essential for differentiation and pathogenicity in *Magnaporthea grisea* (Egan et al., 2007) and *Botrytis cinerea* (Segmüller et al., 2008). These studies revealed putative links between redox signalling and virulence. Both these fungi are filamentous plant pathogens like *F. graminearum* and thus similar signalling mechanism might be expected to control *Fusarium* pathogenesis. To explore pathogenic genes in *F. graminearum*, the current focus has been on targeting MAPK signaling pathways and G protein coupled receptor pathways (Jenczmionka et al., 2003; Rispail and Di Pietro 2009); which are recognized as related to pathogenesis in *M. grisea*. Not much work has been done to investigate redox control on *F. graminearum* virulence and no candidate redox proteins are known at present. Functional analyses of

Fusarium nox mutants are under way, yet there are many gaps to fill at the translational and post translational levels.

2.5 Redox regulation of cellular processes

Highly active oxygen metabolites referred to as reactive oxygen species (ROS) and cellular thiols reflect the redox status of a cell and are frequently implicated with roles in signalling pathways (Klaunig and Kamendulis 2004; Lowell and Shulman 2005). Redox signalling is the process wherein ROS and thiols act as "signals" in biological processes. ROS including superoxides ($O_2^{\cdot -}$) and peroxides (H_2O_2) are generally considered detrimental but unavoidable by-products of aerobic metabolism. Previous studies on ROS focussed on their highly reactive nature and roles in molecular damages such as DNA mutation, lipid peroxidation and protein oxidations. This view was reinforced by investigations on cell aging, apoptosis, pathologies such as cancer, diabetes where ROS are always generated (Paulsen and Carroll 2010). Discovery of specific enzymes like Nox and dual oxidases (Duox) have revealed the flip side of the coin. Positive roles of ROS regulation in cell immunity, cell proliferation, hormone biosynthesis and ion transport (Lambeth 2004; Aguirre et al., 2005) are major findings in animals and plants. In fungi, ROS production is critical for sexual development (Lara-Ortiz et al., 2003), cell differentiation (Osiewacz 2002) and host-pathogen interaction (Nathues et al., 2004). Recently, considerable attention has been focussed on understanding molecular mechanisms of ROS signal reception and transduction in several fungal processes.

2.6 NADPH Oxidases (Nox):

Nox enzymes transfer an electron specifically from NADPH to molecular oxygen to generate superoxide. They have a dedicated function of generating ROS in response to a mixture of chemical, physical, and biological stimuli. Low pH and altered nutritional conditions like nitrogen limitation (Jiang et al., 2011) and intracellular signals such as growth factors, cytokines and calcium mediate Nox activation (Lambeth 2004). Various isoforms of Nox enzymes have been identified in different aerobic organisms. Human phagocytic Nox (Phox) was the first enzyme to be studied. It is composed of two subunits: catalytic and regulatory. The catalytic subunit is gp91phox (Nox 2) and the regulatory subunit consists of p22phox, p47phox, p40phox, p67phox and GTPase RAC (Lambeth, 2004). Nox activity responds as a respiratory burst when phagocytes are exposed to pathogens or inflammatory signals. Other members of Nox family such as Nox1, Nox3-5 have been identified in animal non phagocytes. In *Arabidopsis thaliana*, six different Nox isoforms have been found and are believed to participate in root hair development (Foreman et al., 2003). To date three isoforms of Nox (A, B and C) have been studied in fungal genomes (Aguirre et al., 2005). Specific functions for all isoforms have not been yet determined. An important research area is to investigate explicit functions of Nox enzymes and their regulation in fungal and plant systems.

2.6.1 Fungal Nox enzymes: distribution and function

A varied distribution of *nox* genes is observed among fungi; being absent in yeast and *Ustilago maydis* to all three *nox* genes present in some ascomycota spp like *F. graminearum, M. grisea* and *Podospora anserina.* The occurrence of single *noxA* gene is widely observed, common examples are *Aspergillus nidulans* and *A fumigatus*.

Neurospora crassa, Coprinus cinereus and *Phanerochaete chrisosporium* have both *noxA* and *noxB*. This variation in *nox* gene composition reveals the diversity in morphologies and life cycles of fungal species. Lalucque and Silar (2003) proposed that the *nox* genes evolved in fungi for multicellular differentiation. Occurrence of NoxA within the multicellular fungi strongly supports this hypothesis. Absence of Nox in single celled species like yeast reinforces this idea yet multicellular fungi like *Ustilago* spp provide an exception to the rule. Functional analyses of NoxA and B have shown that these proteins play a key role in controlling fungal cell differentiation, development, pathogenesis and other important biological processes. Nox A triggers the development of fruiting bodies in several sexual species (Lara-Ortiz et al., 2003) whereas NoxB activity is detected in ascospore germination (Malagnac et al., 2004). NoxC is less common and there are no reports of any specific function for this isoform to date (Takemoto 2007).

2.6.2 Structure of fungal Nox:

All Nox isoforms in fungi have six transmembrane spanning domains similar to human gp91phox, forming the "catalytic core". Conserved histidine residues are present in helix three and five, serving to coordinate with two heme moieties, and provide an electron channel across the plasma membrane (Lambeth 2004; Vignais 2002). C-terminus contains four NADPH and two FAD binding sites. NoxA has similar gp91phox catalytic core with no added motifs. NoxB has an extension of 40 amino acids at its N-terminal (Malagnac et al., 2004). N-terminal extension is longer in NoxC, from 170 to 250 amino acids and has an EF-hand motif (supposedly for calcium-binding) as found in human Nox5 (Lewit-Bentley and Rety 2000).

2.6.3 Regulation of Nox complex:

Regulation of fungal Nox is little known as compared to human Nox enzymes. The phagocytic Nox requires assembly of the gp91phox (catalytic subunit), p22phox (act as adapter protein), regulatory subunits and the GTPase Rac2 for activation. The gp91phox and p22phox are membrane protein. In inactive state, the regulatory subunits are cytosolic and GDP-bound Rac2 is coupled with inhibitor protein Rho-GDI in the cytosol (Diebold and Bokoch 2001). In response to exposure to pathogen or inflammatory molecules, regulatory subunits get phosphorylated, permitting the binding of SH3 (Src homology) domain with C-terminus of p22phox at the proline rich region (PRR), and join the catalytic subunit in the membrane. Simultaneous activation of guanine nucleotide exchange factors (GEF) exchanges GDP for GTP on Rac, which facilitates Rac dissociation from Rho-GDI, and transportation to membrane in order to bind with the catalytic domain (Hoyal et al., 2003). A complete assembly of catalytic and regulatory subunits hence activates the Nox complex.

Fungal isoforms have a homologue of p67phox, designated NoxR with similar domain structure at the N-terminal (Takemoto et al., 2006). It includes Rac binding motifs and Nox activation domain, but lacks the C-terminus domains required for binding of p67phox with p47phox and p40phox. Since different domains are found on C-terminus of NoxR, Takemoto et al (2007) proposed that different regulatory components might be involved to interact with NoxR for fungal Nox activation. NoxR is found in all fungal genomes that have NoxA and/or NoxB, advocating that this protein is required for Nox activation. Deletion of the *noxR* regulatory gene from *M. grisea* and *B. cinerea* resulted in mutant phenotypes identical to the *noxA noxB* double mutant suggesting a NoxR

dependent mechanism that activates both NoxA and B (Segmuller et al., 2008). Since the p67phox and/or Rac are not needed for activation of human Nox5 (Kamigut et al., 2005), it has been predicted that fungal NoxC might also have an activation mechanism dissimilar to other *nox*, possibly through calcium signalling.

2.7 Redox mediated control in fungal cellular processes

2.7.1 Cell differentiation:

An increase in cellular ROS level results in increase in antioxidant enzymes such as superoxide dismutases (SOD), catalases, peroxidases etc (Aguiree et al., 2005). In yeast and *Candida albicans*, SOD is critical during the stationary phase (Longo et al., 1996; Lamarre et al., 2001). Multiple catalases (CatA-D) are expressed during different stages of asexual development in *A. nidulans* (Kawasaki and Aguirre 2001). Asexual and sexual development in *N. crassa* occur during a hyperoxidant state (Peraza and Hansberg 2002; Cano-Dominguez et al., 2008); described as a transitory, unbalanced condition in which ROS levels are more than the cellular anti-oxidants (Hansberg and Aguirre 1990). These findings implicate production of ROS and its role as a signal during the process of cell differentiation in fungi.

2.7.2 Sexual development:

Nox is present in all sexually reproducing fungi (Aguirre et al., 2005). Nox A and B have essential roles in sexual development and ascospore germination respectively. *A. nidulans* and *N. crassa noxA* deletion mutants were sterile, produced defective sexual structures and spores incapable of germination (Lara-Ortiz et al., 2003 Cano-Dominguez et al., 2008). Malagnac and coworkers (2004) inactivated two Nox isoforms in the filamentous fungus *P. anserine* (PaNox1 and PaNox2) and showed that they were

required for sexual reproduction and development of infection structures. The germinating ascospore may produce ROS as a signal for hydrolysis of host cell wall and use of nutrients, thus establishing the polarized growth of hyphae. The abundance of antioxidant enzymes in spores and the presence of Nox enzymes in filamentous fungi suggest the decisive role of redox signalling in regulating sexual development in fungi.

2.7.3 Fungal pathogenesis:

Interaction of phytopahogenic fungi with the host plants results in an increase in cellular levels of ROS. It was always believed that the source of ROS was the host plant. However, recent evidence highlights an oxidative burst of fungal origin to start infection process. *M. grisea*, a pathogen of rice blast disease, has very high ROS production associated with development of appressoria. Targeted gene replacement experiments demonstrated that *M. grisea* Nox enzymes are indispensable for pathogenicity (Egan et al., 2007). NoxA and B are required for *B. cinerea* to colonise its hosts (Segmuller et al., 2008) and ergot fungus *Claviceps purpurea* requires its Cpnox1 for pathogenicity (Giesbert et al., 2008). All different roles of Nox discussed earlier, such as in sexual reproduction; development of infection structures; host cellulose degradation and nutrient mobilization, contribute to fungal pathogenesis.

2.7.4 Gaps to fill

ROS induced signal transduction in fungi might follow pathways common to all living systems: histidine/aspartate phosphorylation, G-proteins, MAPK cascade. Cysteine-oxidation in structural and/or regulatory proteins is the other means for ROS regulation (Buchanan and Balmer 2005). ROS modulates protein activity via reversible thiol-oxidation, which targets enzymes, translation initiation factors, elongation factors

and RNA binding proteins. Oxidation of proteins may also be indirect involving an adaptor protein such as peroxiredoxin (Winterbourn and Hampton 2008).

How fungal cell senses ROS as a signal and respond in form of a biological process remains a key question. Understanding of the Nox activation complex is vital to answer such queries. Elucidating how transcription factors and components of a putative MAPK cascade respond to ROS and interact with the Nox complex is underway. The most suitable strategy is to identify the specific targets of Nox generated redox signals in the cell. Ability of ROS to diffuse readily and interact with numerous organic molecules makes it difficult to identify the specific molecular targets. Growing evidence shows that cysteine-rich regions of regulatory as well as structural proteins can be specific targets. There are many questions to be answered and the available genetic and proteomic tools can be useful to comprehend the mechanism behind Nox contribution to redox regulation.

2.8 Perception of ROS by cellular thiols: key to redox signalling mechanism

The thiol group (R-SH) of cysteine is an important target of ROS for oxidation in signalling proteins. Thiolate anion ($RS^{:-}$) is a strong nucleophile that makes thiols one of the most reactive functional groups found in proteins. Immediate oxidation of the SH-group results in a range of products like thiyl radical (-S•), disulfide (-S-S-), sulfenic (-SOH), sulfinic ($-SO_2H$), with increasing oxidation state of sulfur from -2 to +4 and decreasing propensity for reversibility. Prolonged oxidation leads to completely oxidized sulfonic acid ($-SO_3H$) with oxidation state of +6. Since the early oxidations are reversible, thiols act as redox-sensitive switches via thiol- disulfide exchange reactions. This unique reversibility of thiol-oxidation makes cysteine a vital participant in reception and transduction of the ROS signals.

Cysteines are the preferred amino acids for active sites in many proteins because of their ability to form reversible disulfide bonds or to coordinate with transition metals. Disulfide bonds stabilize protein structures and maintain correct protein folding along with regulating activity of enzymes, signal receptors and transcriptional factors. Bestowed with these unique properties, cysteines link the intracellular reduction/oxidation to their responses in the form of biochemical processes in the cell. Transcription factors activated by oxidative stress such as OxyR, RsrA, Yap1p and p53 (Lee at al., 2004a; Kang et al., 1999; Delaunay et al., 2000; Rainwater et al., 1995), molecular chaperons like Hsp33 (Jakob et al., 1999), and enzymes such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and protein kinase A (PKA) (McDonagh et al, 2009) are involved in major mechanisms by which oxidants may participate in signal transduction pathways.

2.8.1 H_2O_2 as a signaling molecule: Cysteine as a sensor

The primary product of Nox activity is superoxide which is readily converted into H_2O_2 by SOD. Among all reactive oxygen species, H_2O_2 is the most abundant (in vivo concentration of 10^{-7} M) and relatively stable molecule (Paulsen and Carroll 2010). Its uncharged nature enables it to diffuse into the cytoplasm as well as across the membranes, and act as paracrine signal molecule. Any increase in H_2O_2 concentration is sensed by cell as a change in redox status. Cysteine-thiols act as "sensors" to this alteration in redox balance due to their enhanced nucleophilic property. Some cysteines are more reactive than others, based on: i) degree of their exposure to oxidants, ii) distance to the nearest free cysteine, and (iii) their pK_a (pKa of a typical thiol group is roughly 8.3, but can vary due to its environment) (Sanchez et al., 2008). Cysteines with a

low pKa are targets for reversible oxidation. This dissimilarity of reactivity among cysteines provides a unique chemo-selective redox control, i.e. specificity for redox-responsive proteins. The early product of the cysteine reaction with H_2O_2 is sulfenic acid (-SOH). An adjacent free cysteine thiol or glutathione will shift the SOH towards the formation of a reversible intra- or inter-disulfide/ mixed disulfide bond respectively. The disulfide can be reduced back to thiol form by either glutathione/glutathione reductase (GSH/GSR) or thioredoxin/thioredoxin reductase (Trx/TrxR) systems. If SOH is not converted into reversible disulfide form, over-oxidation may lead to irreversible thiol states causing protein aggregation and degradation; resulting in physiological and pathological stress conditions. Thus, the switch like nature of disulfides and the specificity of cysteine reactivity provide a regulated mechanism for sensing and transducing the redox signals.

2.9 Detection of Redox-Proteome

McDonagh et al (2009) defined a 'redox proteome' as a subset of proteins susceptible to reversible oxidation at their cysteine residues. Reversible cysteine oxidation is a significant post translational modification (PTM) in enzymatic control and signal transduction; and has been extensively aimed to identify redox-sensitive proteins (LeMoan et al., 2006; Mirzaei et al., 2008; Fratelli et al., 2002). Because of its extremely labile nature, the redox proteome is very challenging to analyse and a combination of proteomic tools is required for its detection. Highly relevant to such analyses is to determine accurately the site and degree of modification, since a protein may have multiple potentially reactive cysteine residues but modification at only a particular site

may result in a functional change. Moreover proteins involved in signal transduction are often low in abundance and not easily detected (Rossignol et al., 2006).

Thiol-specific alkylating reagents such as iodoacetamide (IAM) or N-ethyl maleamide (NEM) which irreversibly bind to free –SH groups while not affecting disulfides permit selective investigation of oxidised cysteines. Florescence probes such as monobromobimane (mBBr) or labelling agents such as biotin or biotinylated IAM/NEM are useful in detecting oxidized cysteine. The specificity of mBBr towards thiols has been extensively employed in many eukaryotic systems (Bykova et al., 2011; González-Andrade et al., 2011). Sophie et al (2009) used 2DE in combination with mBBr to successfully establish a redox-proteome of *Arabidopsis* and identified novel proteins as candidates for redox regulation. Non-gel based proteomic employing biotin-affinity is another approach. In yeast, key metabolic enzymes are demonstrated to undergo reversible thiol-oxidation after exposure to H_2O_2 (Mc Donagh 2009). A similar principle was employed in methodologies like "thiol trapping technique" (Leichert and Jakob, 2004) and "biotin-switch method" (Jaffrey and Snyder, 2001) to study reversible thiol-oxidation causing inactivation of tyrosine phosphatases and S-nitrosylation respectively. Most importantly, mBBr label or biotin-tag not only allows identification of redox sensitive proteins but also facilitates assigning the site of redox modification within each protein.

2.10 Proteomic tools

Mass spectrometry (MS) is an exceptionally suitable tool for identifying proteins and studying post translational modifications. MS following protein separation methodologies like two dimensional polyacrylamide gel electrophoresis (2-DE) or liquid

chromatography (LC) provide an effective and sensitive approach to discover proteins in complex mixtures. Introduction of soft ionization techniques like electrospray ionization (ESI) is one of the major advances in mass spectrometry (Fenn et al., 1989). Peptides are non-volatile and heat labile and ESI enables the ionization of a peptide into gas phase without significant damage.

2.10.1 Two-Dimensional Gel Electrophoresis (2-DE)

2-DE is the conventional proteomics tool for obtaining a global view of a protein profile. The proteins are first separated according to their isoelectric points (pI) and subsequently based on their relative molecular mass (Mr). Use of immobilized pH gradient (IPG) strips has further enhanced the protein separation by allowing wide range pH gradients. 2-DE is advantageous as the gels permit simultaneous examination of overall protein profiles, and comparison of relative abundances and/or changes in expression levels. Relevant information about a protein such as pI values, Mr, solubility, isoforms, PTMs etc. can also be gathered using this method (Gorg et al., 2004). However, all of the proteins possibly forming the proteome cannot be observed by 2-DE owing to its limitations. These include poor gel-to-gel reproducibility, difficulty handling low abundant proteins, hydrophobic proteins and proteins with extreme pI and Mr; and the technique is labour intensive. Thus the protein profile observed by 2-DE is apparently a biased subset of proteins depending on their abundance and solubility in extraction buffer.

2.10.2 Shot-gun: Non-Gel Approach

Shot-gun proteomics integrates separation of tryptically digested peptides through LC followed by protein identification with mass spectrometry. The main advantages are

that LC–MS can be automated and it provides higher resolution of complex peptide mixtures (Zhu et al., 2010). Development of 'multidimensional protein identification technology' (MudPIT) is a major advancement since peptide separation is directly coupled with ionization in a mass spectrometer, resulting in extreme sensitivity (Washburn et al., 2001; Wei et al., 2005). Shot-gun approach can identify low abundance and/or membrane proteins also; thus providing an improved and unprejudiced approach to look at subtle cellular changes, like redox modifications. However higher cost of reagents, requirements of specific software and availability of sequenced genomes for MudPIT method makes 2-DE still the most common proteomic tool especially in non-model systems like *F graminearum*.

Given the intricacy of a proteome and subtlety of redox signalling processes, no single proteomic technology can answer all questions. For our study, integrating both gel and non-gel based proteomic tools offered a global yet detailed view and allow thorough investigation of redox control during biological processes. Combining cysteine labelling methods can help precise cysteine mapping in redox regulated proteins.

2.10.3 Quantitative MS

Modern proteomics includes a discovery phase, typically conducted by mass spectrometry (MS) to identify proteins; followed by validation and quantitation phase, to accurately measure protein changes. Stable isotope labelling (Gygi et al., 1999), label-free methods (Fang et al., 2006) and targeted proteomic techniques such as selected ion monitoring (SIM) (Yun et al., 2011) and selected reaction monitoring (SRM) (Kitteringham 2009), are various quantitative approaches used nowadays. Fundamentally protein quantification is carried out in two ways. In first, the changes in ion intensity are

measured as peptide peak areas or peak heights in chromatograms. The second way is based on spectral counting of identified proteins after MS/MS analysis (Zhu et al., 2010; Chen and Yates, 2007).

SIM is a useful scanning technique to validate known peptides in a given sample when an ion trap mass spectrometer is used for analysis (Jorge et al., 2007). In SIM mode, a specific mass-to-charge ratio (m/z) range is detected as opposed to the full spectrum range. The mass spectrometer is set to collect data at only the m/z of interest and only compounds with the selected mass fragments are recorded. Since more time is spent looking at specific m/z, it increases sensitivity. Also SIM allows the collection of more points across a chromatographic peak, thus enhancing the accuracy and precision of quantitative results. SIM mode has been employed in pharmacokinetic studies for determination of drugs in human plasma (Yun et al., 2011; Ding et al., 2002) as well as for quantitation of small organic compounds (Calabrese et al., 2009). Scanning techniques were shown useful to target low abundance proteins in *Arabidopsis* (Wienkoop and Weckwerth, 2006) and quantify high to medium abundance proteins in human plasma (Anderson and Hunter 2006). Alternatively, SRM-based approaches were used to identify and quantify phosphorylation sites in key regulatory proteins (Unwin et al., 2005; Mayya et al., 2006) and investigate the cell signalling networks (Wolf-Yadlin et al., 2007).

Triple quadrupole mass spectrometer is considered ideal for quantitation attributed to its excellent sensitivity, high duty cycle, high dynamic cycle and increased mass accuracy, as compared to linear ion trap. However, ability for MS^n fragmentation still makes ion trap suitable for quantification studies. Recently, researchers have

successfully used linear ion trap for analysing large peptides with improved detection limits (Shipkova et al., 2008) and absolute quantification of myoglobin to a concentration of 460 pg/microL in human serum (Mayr et al., 2006). In another study, Lin and group (2006) reported quantification of medium abundance serum proteins by applying SRM in a single-quadrupole linear ion trap with high precision, at ~1–30 μg/mL levels. These studies open a window for researchers using ion trap MS to perform validation and/or quantitation; provided only a few target compounds have to be assessed and mainly at nanoscale level.

MATERIALS AND METHODS

Chapter 3: Material and methods

3.1 Biological material and culture conditions

F. graminearum strain Fg 233423 (WT) and mutant *ΔnoxAB* were graciously provided by Dr R. Subramaniam, ECORC-AAFC, Ottawa, Canada. *ΔnoxAB* is a deletion mutant for two genes *noxA* and *noxB*. Fungal cultures were grown on synthetic nutrient agar plates under an alternating 12 h, 25 °C light/12 h, 22 °C dark cycle for 7 days. Agar plugs of fungal cultures were stored in 10% (v/v) glycerol at -80°C for long term use. Spores were produced by adding the agar plugs to liquid carboxy methyl cellulose (CMC) medium previously sterilized by autoclaving for 20 min at 121 ºC. After incubating for 5 days at 28°C the culture was filtered through three layers of cheese cloth to obtain a spore suspension. Fungal spores were counted using a hemocytometer and adjusted to 5 x 10^4 conidia per millilitre using sterilized distilled water.

A two-stage media protocol as described by Harris et al (2007), modified from Miller and Blackwell (1986), was employed to induce DON production in liquid culture. Approximately 100,000 spores were inoculated into 100ml of first stage medium (3 g NH_4CL, 2 g $MgSO_4 \cdot 7H_2O$, 0.2 g of $FeSO_4 \cdot 7\ H_2O$, 2 g KH_2PO_4, 2 g peptone, 2 g yeast extract, 2 g malt extract, 20 g glucose in 1 L distilled water) and grown at 25 °C on a rotary shaker at 180 rpm for 24 hours. This culture was then transferred to second stage medium (1g $(NH_4)_2HPO_4$, 3 g KH_2PO_4, 0.2 g $MgSO_4 \cdot 7\ H_2O$, 5 g NaCl, 40 g sucrose, 10 g glycerol in 1 L of distilled water) and grown for next 24 h. Fungal tissues were harvested at 4°C, partially dried by filtering through 1mm Whatman filter paper with a Buchner funnel, immediately frozen in liquid nitrogen and stored at -80C.

3.2 Protein extraction

Three biological replicates of both WT and *ΔnoxAB* cultures were grown for comparative proteomic analysis in both gel and non-gel experiments. Harvested fungal tissues were ground in liquid nitrogen using a mortar and pestle. Ground tissue was suspended in 10 volumes of protein extraction buffer (10% (w/v) TCA in cold acetone) and incubated at -20°C for 3h. The samples were centrifuged at 14,000g for 20 min at 4°C. The pellet was washed with 8ml of ice-cold acetone and centrifuged as before. After repeating five acetone washes, final pellet was dried under a stream of nitrogen gas.

3.3 mBBr labeling and Gel Electrophoresis

3.3.1 Protein alkylation, reduction and mBBr labeling

Selective labeling of redox reactive cysteine(s) with mBBr (Calbiochem) was achieved as depicted in Figure 3.1. Dried protein extracts were dissolved in 2ml of denaturing buffer (8 M Urea, 4% (w/v) CHAPS, 2 mM EDTA and 50mM Tris–Cl pH 8.0) with 2mM PMSF and 100 mM IAM and shaken in dark for 30 min. Samples were then reduced with 40 mM of DTT for 45 min on a rotator at room temperature. Excess DTT was used to quench unreacted IAA along with complete reduction of disulfide bonds. Samples were then centrifuged at 14,000g for 60 min to obtain protein extracts. The protein extract was precipitated with TCA/acetone and washed with ice cold acetone to remove remaining DTT. The pellet thus obtained was dried under a stream of nitrogen gas and stored at -80°C. Dried protein extracts were re-dissolved in SDS sample buffer (for 1-DE) or isoelectric focusing (IEF) solution (for 2-DE). For 1-DE, 10 µg proteins per lane in equal volumes were loaded on 4% stacking gel (pH 6.8) and 12% resolving gel (pH 8.8). The gel electrode assembly was placed in a Mini PROTEAN II

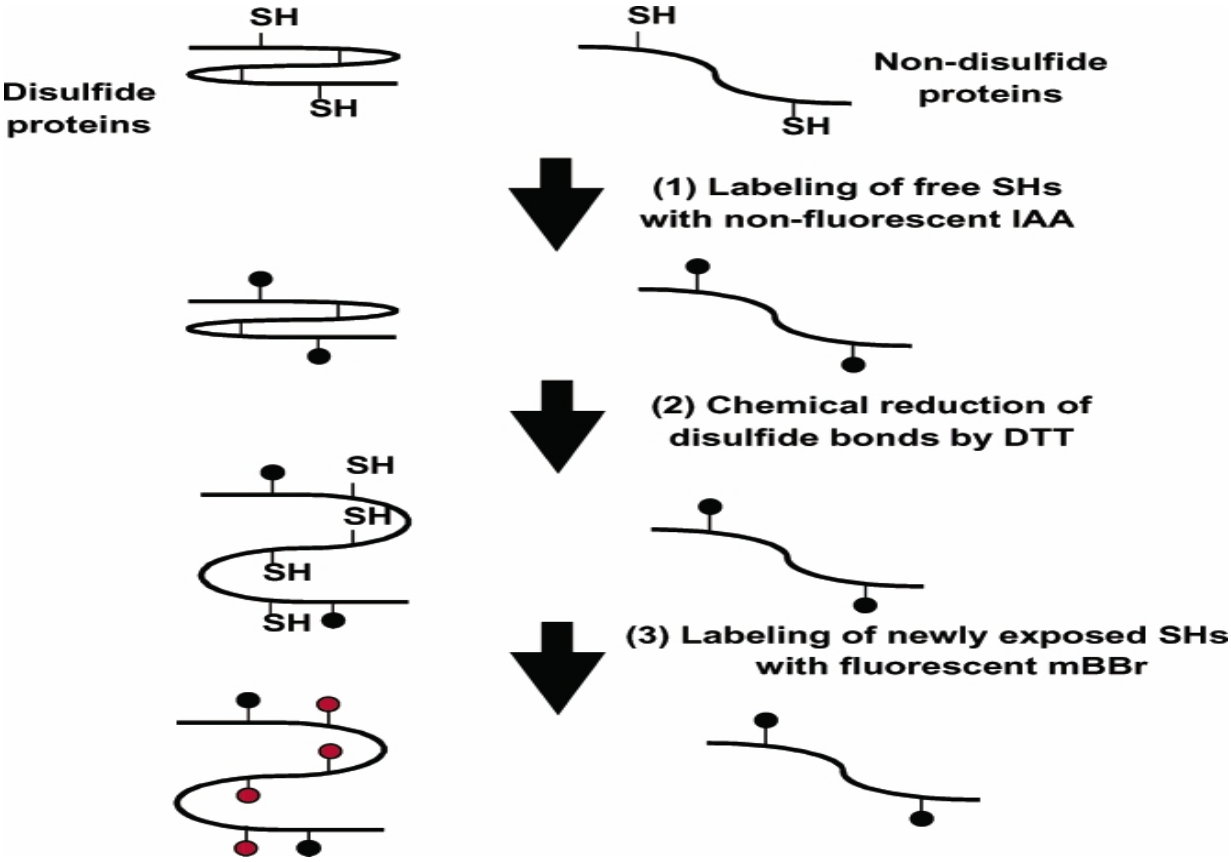


Figure 3.1 Schematic protocol for selective labeling of reversibly oxidized cysteines with mBBr. Black dots: Native free thiols alkylated with IAA; carbamidomethylation (CAM) modification. Red dots: mBBr labeled thiols made available after reduction of disulfide groups with DTT; bimane modification.

electrophoresis chamber (BioRad, Hercules CA) and proteins were separated at 100V until the dye band moved completely to the bottom.

For 2-DE, proteins were re-dissolved in 1ml of IEF solution containing 8 M Urea, 4% (w/v) CHAPS, 1% (w/v) ampholyte (Biolyte 3-10, Bio-Rad). Proteins were quantified using Bradford dye-binding assay (Bio-Rad) with BSA as standard. 500 µg proteins were labeled with 2mM mBBr and vortexed in dark for 30 min at room temperature (protocol modified from Bykova et al., 2011; Yano and Kuroda, 2006). Final protein volume was adjusted to 450 µl with IEF solution containing traces of bromophenol blue. To get rid of fine particulate matter, the solution was centrifuged at 100,000g for 30 min on an ultracentrifuge (Airfuge, Beckman Coulter). Labelled proteins were then used to rehydrate 24 cm IPG strips with 4-7 pH range (GE Healthcare) overnight at 20°C. Rehydration was done in a Teflon reswelling tray with strips placed under a layer of mineral oil (Dry Strip Fluid, GE Healthcare).

3.3.2 First dimensional isoelectric focusing

Focusing was conducted for 58.3 kVh on a Multiphor II IEF unit (GE Healthcare) with current maintained at 50 mA per strip throughout the run (Rampitsch et al., 2006). Focused strips were washed twice with equilibration buffer (50mM Tris-Cl pH 8.8, 6 M Urea, 30% (v/v) glycerol, 2% (w/v) SDS) with 1% (w/v) DTT for 8 min each. Same equilibration was followed twice with buffer containing 2.5% IAM instead. Finally the strips were rinsed with electrode buffer (25mM Tris, 192 mM glycine and 0.1% (w/v) SDS).

3.3.3 Second dimensional SDS-PAGE

Clean and lint-free glass plates were assembled in the pouring apparatus according to the manufacturer's instructions. 10% (w/v) APS was freshly prepared and TEMED reagent was used directly as commercially provided. 12% non-gradient acrylamide solutions were prepared. After pouring, the gels were overlaid with water-saturated 2-butanol and left to polymerize for 2 hours. IPG strips were placed on polymerized gel surface and the glass plates were assembled into Ettan Dalt-6 electrophoresis unit (GE Healthcare). The gels were electrophoresed in Tris-glycine buffer (25mM Tris, 192 mM glycine and 0.1% (w/v) SDS), with a power gradient of 2W per gel for 90 min followed by 17W per gel until the bromophenol blue dye front had left the gel.

3.3.4 Visualization and analysis of redox sensitive thiol-status v/s total proteome

Gels were fixed in 40% (v/v) methanol/10% (v/v) acetic acid for 2 h to remove unbound mBBr and reduce the background. The florescence of mBBr-derivatized proteins was visualized using ultraviolet transilluminator at 365nm. To study the total proteome, gels were stained overnight with 0.1% (w/v) Coomassie brilliant blue G-250 in 12.5% (w/v) TCA. Stained gels were scanned on a laser scanner (Molecular Imager FX: Bio-Rad) using a 532nm laser for excitation and a 550nm long pass filter. The fluorescence images and Coomassie stained images of WT and *ΔnoxAB* proteomes were compared. The differentially fluorescing spots in WT and *ΔnoxAB* proteomes were identified and examined in total proteomic profiles of both strains. The protein spots only fluoresced in WT but present in both proteomes were excised.

The intensity of mBBr florescence is proportional to the number of reduced thiols available in the protein for the reaction. The degree of mBBr reaction with available thiols was quantified using densitometry with Quantity One imaging software (Bio-Rad). Similarly the Coomassie stained gel images were quantified using the same software to compute the total protein content. The florescence level to protein expression level ratio reflects the number of reduced cysteines available as compared to the total amount of protein (Bykova et al., 2011). This ratio for a particular spot was assessed by dividing its intensity of mBBr signal on florescent image by its intensity on the corresponding Coomassie stained gel image. Matched pair comparisons were performed to determine if there were any significant ($p < 0.05$) differences in the total protein contents of the WT and *ΔnoxAB* in these gels spots. For the same protein spot in three replicate gel images, the mean values and standard deviations were calculated. All statistical analyses were performed using statistical software (JMP Ver 8.0.1, SAS Institute Inc., 2009).

3.3.5 In-gel digestion

Protein spots of interest were excised from gels, cut to pieces of 1 mm^2 and digested *in situ* with tryspin (Promega, modified, sequencing grade). Briefly, trypsin digestion buffer (100 mM NH_4HCO_3, 10 % (v/v) acetonitrile (ACN), 2.5 mM $CaCl_2$) was added to cover the gel pieces and left for 30 min on ice. Trypsin concentration in the digestion buffer was adjusted to12 ng/µl. Samples were incubated at 37°C overnight for digestion. Peptides were extracted from the gel through vortexing and centrifugation in a series of formic acid (FA)/acetonitrile (ACN) (v/v) extraction solutions as follows: 5% FA, 1% FA/ 5% ACN, 1% FA/ 60% ACN, and 1% FA/ 99% ACN. The collected

supernatants which contained the peptide mixtures were dried in a Speed Vac and peptide mixtures were analyzed by LC-MS/MS.

3.4 Biotin-affinity chromatography and Shot Gun proteomics

3.4.1 Alkylation, reduction and biotinylation of redox sensitive cysteine residue(s)

Figure 3.2 depicts the outline of the protocol for detection of reversibly oxidized cysteine containing proteins. Dried protein extracts obtained after TCA/Acetone precipitation (described before) were dissolved in 2ml of denaturing buffer (8 M Urea, 4% (w/v) CHAPS, 2 mM EDTA and 50mM Tris–Cl pH 8.0) with 2mM PMSF and 100 mM IAM and shaken in dark for 30 min. Samples were then reduced with 40 mM of DTT for 45 min on a rotator at room temperature. Excess DTT was used to quench unreacted IAA along with complete reduction of disulfide bonds. Samples were then centrifuged at 14,000g for 60 min to obtain protein extracts. The protein extract was precipitated with TCA/acetone and washed with ice cold acetone to remove remaining DTT. The pellet thus obtained was dried under a stream of nitrogen gas and stored at -80°C. Dried protein extracts were re-dissolved in denaturing buffer (8 M Urea, 4% (w/v) CHAPS, 2 mM EDTA and 50mM Tris–Cl pH 8.0) containing 0.5 mM biotin-HPDP (Pierce Biotechnology) and incubated on a rotator in the dark for 45min. Samples were precipitated with TCA/acetone and 5 washes with cold acetone were given to remove unbound biotin-HPDP. Pellets were redissolved in 1ml of 50mM ammonium bicarbonate. The protein samples were desalted and concentrated to 500 µl by centrifuging at 4,000 g for 30 min, at 22°C in 2ml spin-column (Vivaspin, Viva Scientific, UK) with a 5 kDa cut-off. To get rid of fine particulate matter, the solution was centrifuged at 100,000g for 30 min on an ultracentrifuge (Airfuge, Beckman Coulter). Proteins were quantified using

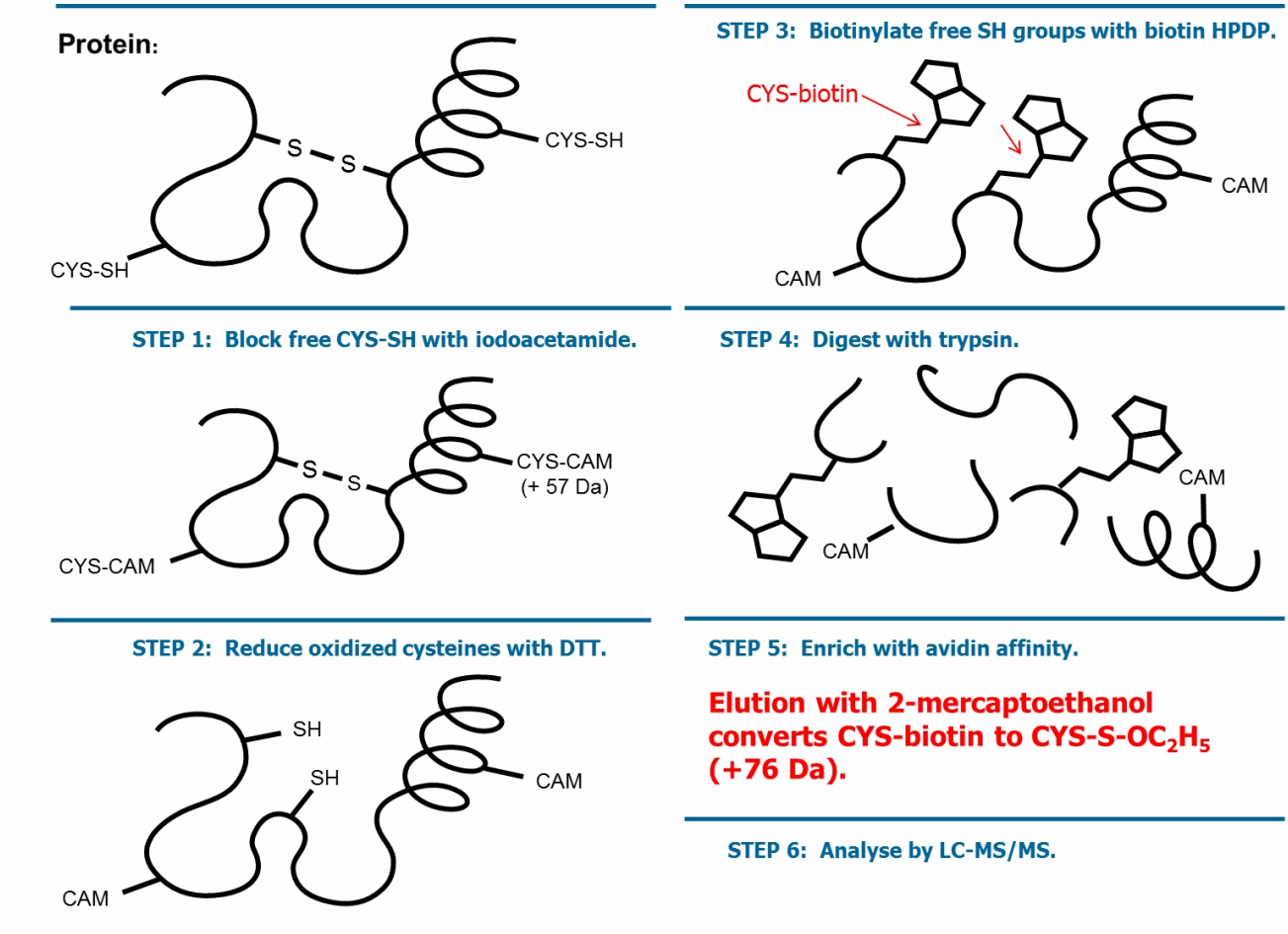


Figure 3.2 Outline of protocol for detection of reversibly oxidized cysteines using biotin-affinity chromatography followed by LC-MS/MS.

Cys-SH: Native free thiols; blocked with IAM to modify into Cys-CAM: carbamidomethylation modification (+57Da)

Cys-Biotin: DTT reduced free thiols convert into Cys-Destreak modification (+76Da) after elution with 2-mercaptoethanol from affinity column.

the Bradford dye-binding assay (Bio-Rad) with BSA as standard. 500 µg of protein was tryptic digested (Promega) at a ratio of 1:100 (wt:wt) trypsin:protein and incubated at 37 °C overnight.

3.4.2 Enrichment of redox-subpeptidome

Streptavidin–Sepharose CL-4B resin (Sigma-Aldrich) was prepared in 2ml capacity resin columns (Thermo Scientific) by washing twice in binding buffer (4 M Urea, 2% CHAPS, 50 mM NaCl and 25 mM Tris–Cl, pH 8.0). Peptides were incubated in resin (according to manufacturer's instruction) for 3h at room temperature. Post incubation, the resin was washed once with binding buffer, twice with wash buffer A (8 M Urea, 4% CHAPS, 1 M NaCl and 25 mM Tris–Cl, pH 8.0) and three times with wash buffer B (8 M Urea, 4% CHAPS and 25 mM Tris–Cl, pH 8.0). In order to remove urea, the resin was washed four times with wash buffer C (5 mM ammonium bicarbonate/20% acetonitrile). All wash steps were carried out with volumes equal to resin column volume and by centrifuging at 500 g for 1 min each.

3.4.3 Elution of biotinylated peptides

For elution of biotinylated peptides, 170 µl of wash buffer C containing 5% (w/v) β-mercaptoethanol was added to the resin, with bottom of the column sealed and incubated at 90°C for 3min. Elution was carried out at speed of 500 g for 1 min and eluent was collected in a fresh tube.

3.5 LC-MS/MS analysis

Tryptic peptides from 2-DE gels or biotin-affinity chromatography were analyzed in a LTQ linear ion trap mass spectrometer (Thermo Finnigan, San Jose, CA) connected on-line to a nano-HPLC (Dionex UltiMate^{TM}3000). A 10cm C_{18} column (5µm particle/

300Å pores) was prepared in-house and used to introduce peptides into the mass spectrometer via nanospray ionization at 250 nL/min using a 2–80% v/v ACN gradient in 1% v/v formic acid, 0.5% v/v acetic acid over 65 min. The mass spectrometer was operated in positive ion mode with source temperature 200 °C and tuned in nano-spray mode using 10 μM (Glu-) Fibrinopeptide B (GluFib) singly charged ion at m/z 1552.67. A full survey scan (MS) was acquired over 400– 2000 m/z range. MS/MS data were acquired in data dependent scan mode, using a "Big Five" program which selects the five most intense ions for fragmentation, with dynamic exclusion set to 20 sec. In all cases, a nESI spray voltage of 1.8 kV was used.

To check whether peptides of interest were absent from samples, rather than missed due to the nature of the data-dependent "Big 5" scan, samples were re-analyzed for selected peptides using the mass spectrometer in SIM mode. SIM mode is designed to scan for selected precursor ions with m/z values of the peptides in question. The linear ion trap was programmed to scan mass windows of ± 1.5 m/z units in appropriate time segments, based on the retention time of the peptide of interest, during the chromatographic run. SIM chromatograms were generated showing the presence or absence of the precursor ions of interest. The peptides, their precursor ion m/z values and retention times are given in Table 3.1.

3.6 Database search and assignment of redox sensitive cysteines

CID fragmentation spectra were queried against *F. graminearum* protein database using MASCOT (v2.2, Matrix science, UK). Proteins were then identified through homology search with BLASTp against the non-redundant sequence database at the National Centre for Biotechnology (NCBI). Peptide mass tolerance was set to ±2 Da,

Table 3.1 Precursor Ions monitored in SIM mode at respective retention time (RT)

Protein (peptide sequences)	m/z	Charge	RT (min)
FG01246.1 Protein disulfide isomerase (K.SNDLVLAEFFAPWCGHCK.A)	724.20	3+	50.31
FG04558.1 Carbonic anhydrase (R.IPAEQICGLEPGEAFIHR.N)	684.31	2+	40.88
FG06847.1 14-3-3 protein homolog (K.ICEDILEVLDQHLIPSAK.S)	704.98	3+	50.93
FG07528.1 Pyruvate kinase (K.RPVSTVESCAMAAVR.A)	826.45	3+	55.11
FG08077.1 Putative NADPH dehydrogenase (R.LFLAPLCQYSAK.D)	715.03	3+	39.73
FG10737.1 Adenylate kinase 1 (K.ECQGGFILDGFPR.T)	757.05	2+	43.50
FG10905.1 40S ribosomal unit (M.APSNLPSVMNATSQDIETLLAAQCHLGSK.N)	1025.10	3+	53.87

fragment mass tolerance was ±0.8 Da, and up to 1 missed tryptic cleavages were allowed. Monoisotopic peptides with charged states of +1, +2 and +3 were queried using using b- and y-ion series employing automatic decoy searches.

Peptides from 2D gels were searched with MASCOT MS/MS ion search parameters using bimane (C), carbamidomethyl (C) and oxidation (M) as variable modifications. Carbamidomethyl modification (+57 Da) is due to thiol-alkylation reaction. The "bimane" modification was incorporated into MASCOT ion search with elemental composition $C_{10}H_{10}N_2O_2$ and monoisotopic mass 190.07 for covalent bimane adduct to Cys residue. Peptides eluted from biotin-affinity column were queried using β-mercaptoethanol modification of cysteine residues called as "destreak" (C) (+76 Da), carbamidomethyl (C) (+57 Da) and oxidation (M) (+16 Da) as variable modifications. β-mercaptoethanol reduction of cysteine-biotin bond brings a modification of +76 Da at cysteine residue and was incorporated into MASCOT ion search with elemental composition of C_2H_4SO. With ion score >95% or higher, MS/MS was considered significant. MS/MS fragmentation was manually examined when a protein with significant score in 2 replicates was found to have a lower score in 3rd replicate to ensure a high quality match.

RESULTS

Chapter 4: Results

4.1 Gel electrophoresis:

4.1.1 Selective labeling by mBBr

We employed the thiol-specific mBBr labeling procedure to compare the redox disulfide proteomes of *F. graminearum* WT against Δ *noxAB*. To confirm the selective labelling of reversibly modified thiols by mBBr, Fg WT and Δ *noxAB* protein extracts were subjected to only alkylation, alkylation and reduction, and only reduction prior to labeling. Labeled proteins were then resolved in 1D-SDS PAGE gel. Lane 1 of Figure 4.1 A shows that the least florescence was observed when protein thiols were blocked with IAM, without further reduction of disulfides. Selective proteins gave mBBr signals when labeled after alkylation and reduction (lane 2) as compared to all reduced thiols (lane 3). The Coomassie blue stained images of the same gels demonstrated that not all proteins were labelled with mBBr thus further establishing the use of thiol-specific florescence to distinguish redox disulfide proteome (Figure 4.1 B).

4.1.2 Differential expression of redox disulfides

Labeled and total proteomic maps (pH 4-7) of samples from the two strains were obtained employing the mBBr labeling procedure with 2-DE gels. Three biological replicates, with three technical replicates each, were run for each strain to assess reproducibility. Figure 4.2 shows the florescent images of reversibly oxidised proteins as a consequence of Nox activity that is termed “redox proteome”. Gels were stained with Coomassie Blue to obtain the total proteomes of wild type and mutant (Figure 4.3). Comparative analysis of WT and mutant redox profiles was done to examine the differences in thiol status in each strain as a consequence of Nox activity.

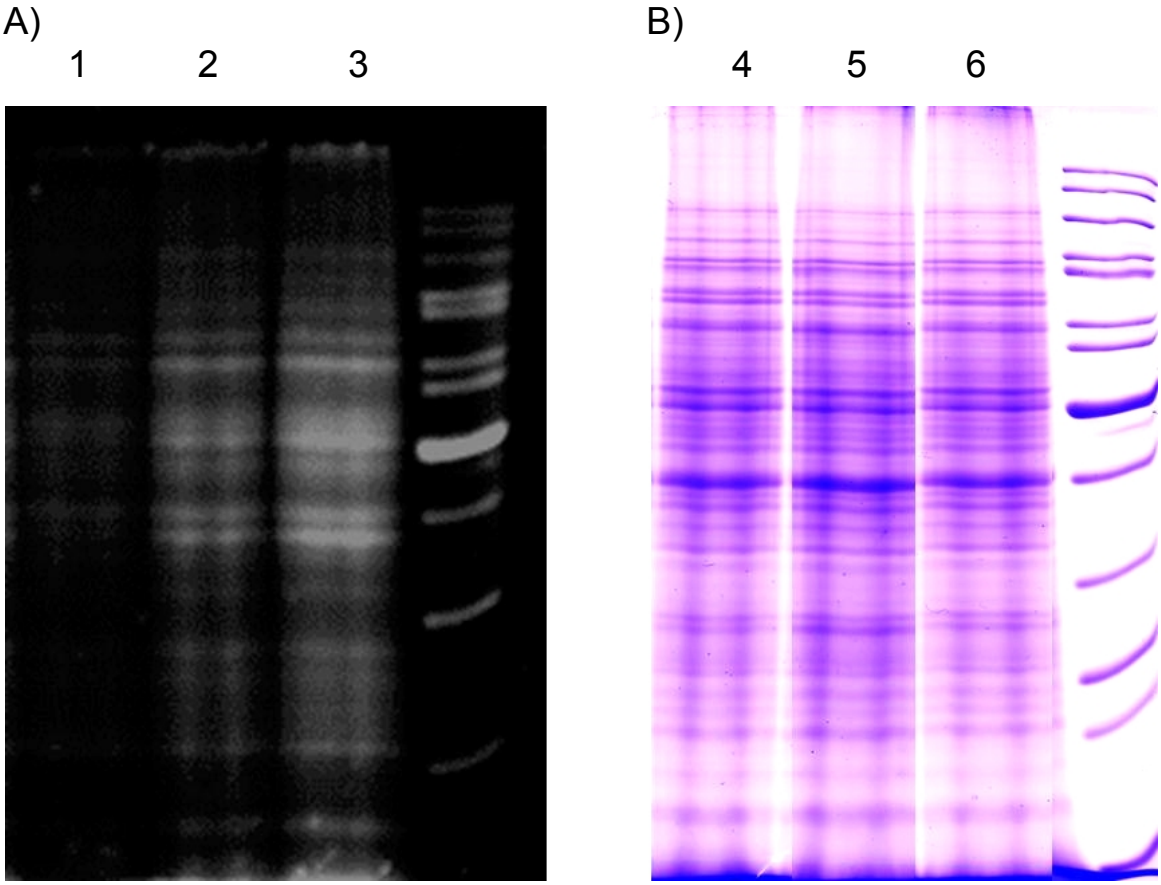


Figure 4.1 Selective labeling by mBBr in proteins extracted from wild type *F. graminearum*. A). Proteins labeled with mBBr (lanes 1, 2 and 3). B). Proteins stained with Coomassie blue (lanes 4, 5 and 6).

Lanes 1 and 4: proteins only alkylated and labeled.

Lanes 2 and 5: proteins alkylated, reduced and labeled (complete mBBr procedure).

Lanes 3 and 6: proteins only reduced and labeled.

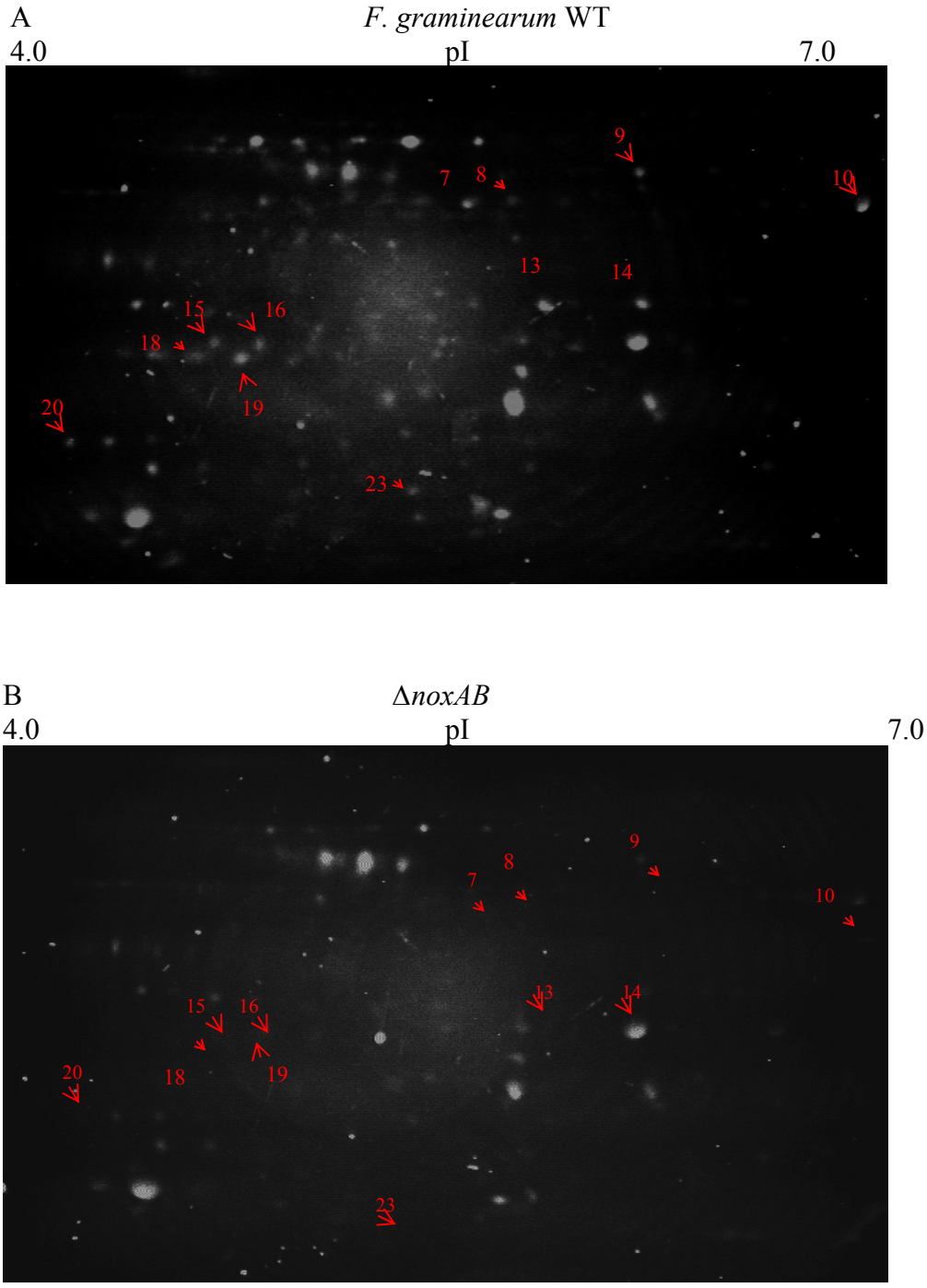


Figure 4.2 Protein thiol redox state in *Fg* wild type and Nox enzyme mutant Δ*noxAB*. Representative 2-DE UV images of mBBr labeled 500 µg protein extracts from WT (A) and ΔnoxAB (B) in pH range 4-7. Protein spots indicated by arrows were analysed by LC-MS/MS.

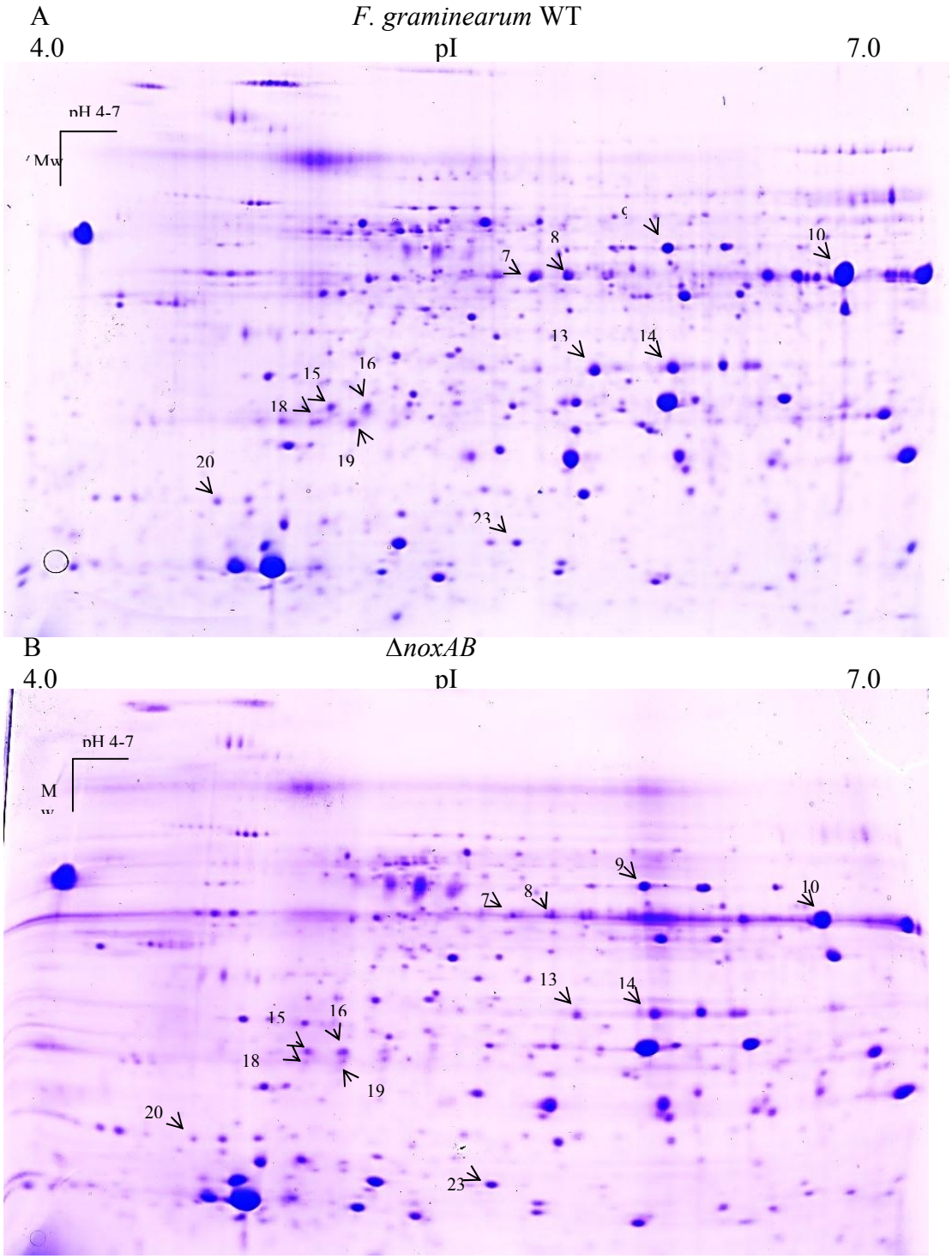


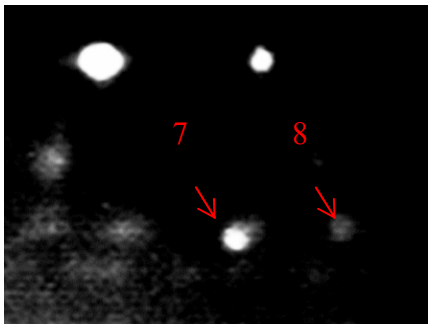
Figure 4.3 Total protein profile of *Fg* wild type and Nox enzyme mutant Δ*noxAB*. Coomassie stained 2-DE images of 500 µg protein extracts from WT (A) and ΔnoxAB (B) in pH range 4-7. Protein spots indicated by arrows were analysed by LC-MS/MS.

A total of 12 protein spots were consistently detected with differential florescence patterns between the two strains (Figure 4.4). They gave mBBr signals in WT but not in mutant strain. They were expressed in total proteomes of both WT and Δ*noxAB*, thus representing the pool of proteins oxidised exclusively due to Nox activity. For all 12 spots, the total protein content did not differ significantly between WT and Δ*noxAB* at $p<0.05$ (Figure 4.5). The florescence to total protein ratio in WT varied from 0.31 to 2.62 with a mean of 1.49 and standard deviation of 0.66 (Table 4.1), whereas the florescence intensity in mutant was found to be zero for all of these 12 spots.

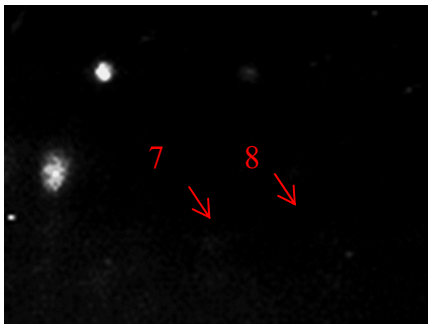
4.1.3 Identification of bimane labeled proteins

Peptides extracted from 12 gel spots showing reproducible florescence differences were analyzed by LC-MS/MS. Along with the putative identity of the proteins detected, the coverage of the amino acid sequence with significant peptides, total MASCOT score and individual ion score, number of peptides, and the assigned site of cysteine modification for the identified protein are presented in Table 4.1. From 12 spots, 10 proteins were identified containing modified cysteines. The calculated molecular mass of the identified proteins ranged from approximately 13 to 70 kDa. Identification of isoforms from a row of spots with different pI and the same molecular weight is a common observation in 2D gels, which likely results from posttranslational modifications. This is likely the case for spot # 7 and 8: Fructose-1, 6-bisphosphate aldolase (FG02770.1); spot # 13 and 14: Protein similar to mannitol dehydrogenase (FG04826.1) and spot # 15 and 16: Cytochrome c oxidase subunit 4 (FG02712.1). The same peptides containing bimane labeled cysteines in isoforms of these three proteins were found; C_{12} in spot # 7 and 8, C_{27} in spot # 13 and 14 and C_{10} in spot # 15 and 16.

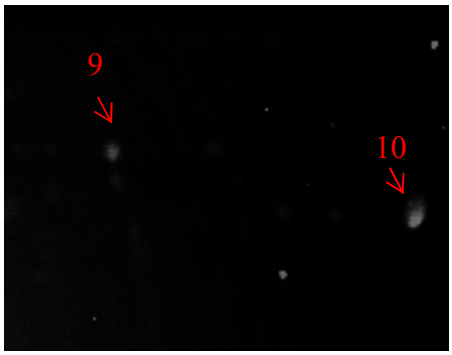
A



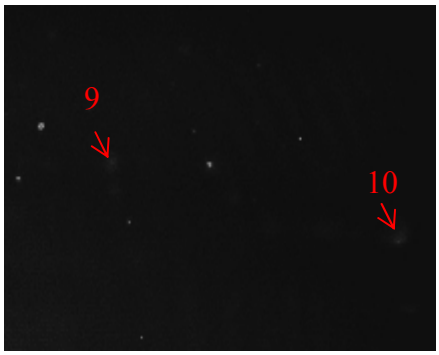
B



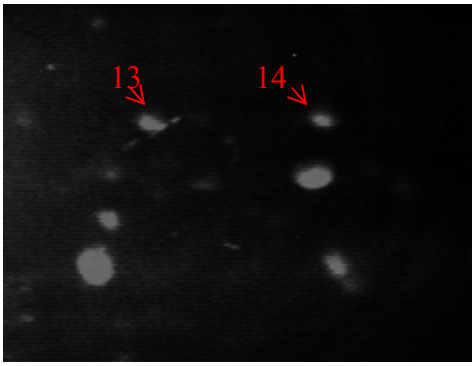
C



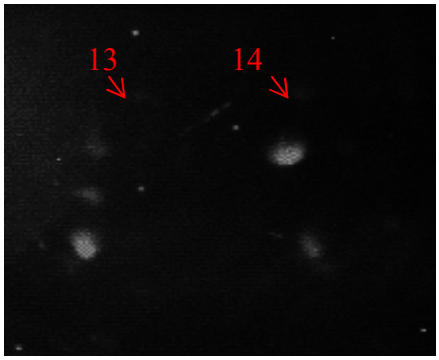
D



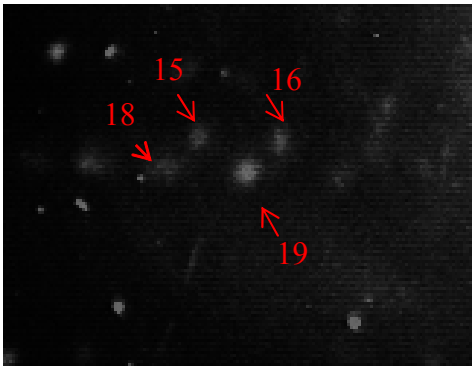
E



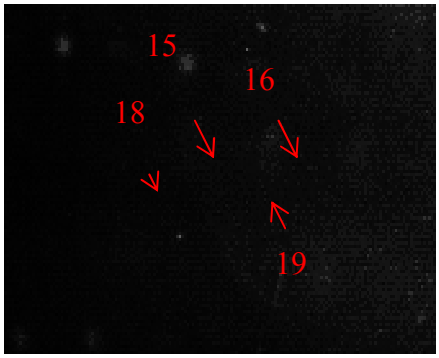
F



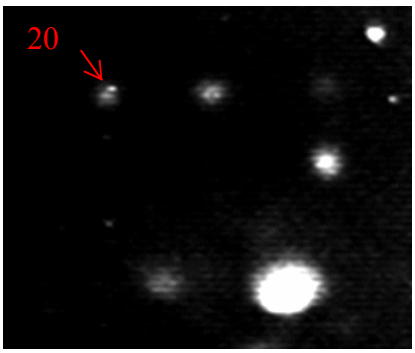
G



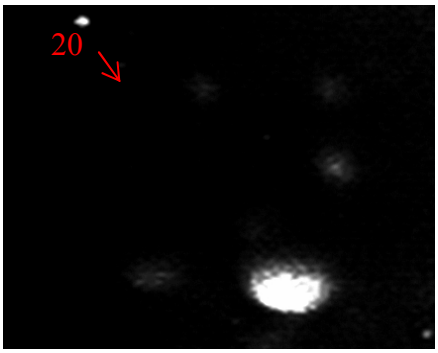
H



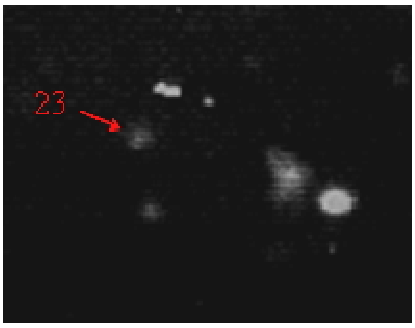
I



J



K



L

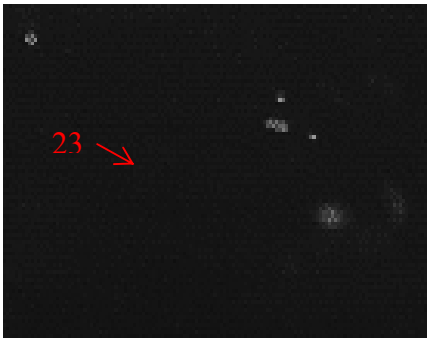


Figure 4.4 Close up of different thiol-redox status of proteins in *Fg* wild type (A, C, E, G, I and K) and Nox enzyme mutant Δ*noxAB* (B, D, F, H, J and L) shown by spot numbers 7, 8, 9, 10, 13, 14, 15, 16, 18, 19, 20 and 23. Protein spots fluoresced only in WT and indicated by arrows were analysed by LC-MS/MS (Table 4.1).

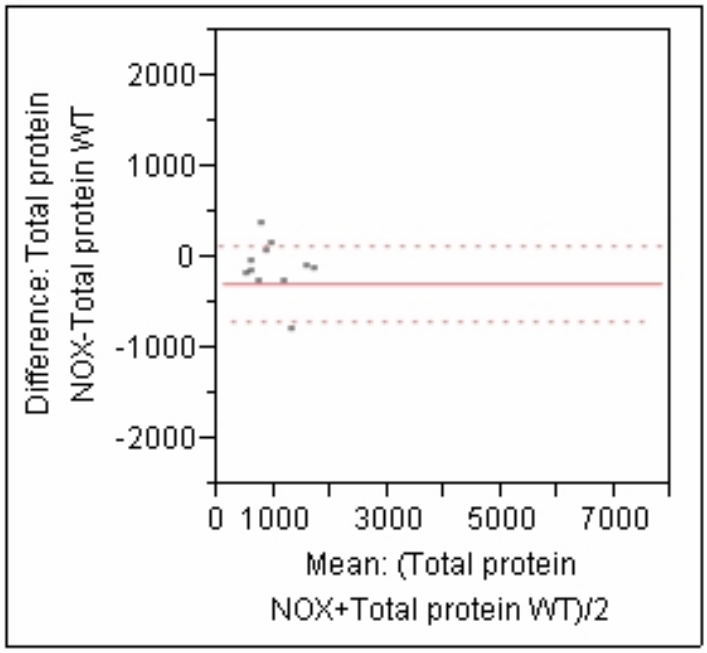


Figure 4.5 Matched paired comparisons of the total protein content for 12 gel spots which show differential thiol-redox status in WT and Δ*noxAB* protein extracts (Figure 4.4). The probability (> t) = 0.939 is much more than 0.05 indicating that the gel spots did not differ in their total protein content between two strains. The graph shows that the difference between the total protein contents of each spot is close to the solid red line indicating the differences are close to zero and are not significantly different from zero.

Table 4.1 Identification of differentially fluoresced proteins in *F. graminearum* wild-type and Δ*noxAB* , by LC-ESI-MS/MS and MASCOT, using 2D gel approach

Spot[1]	Putative identity[2]	ID[3]	P/C[4] Score[5]	Cysteine peptides[6] Ion Score[7]	Modified Cys[8]	Flo:Total Protein[9] Mean±Std Dev
7	Fructose-1,6-bisphosphate aldolase	FG02770.1	63% (13) 4633	K.VNVDTDMQFAYCSGIR.D (85) R.SLAPTYGIPVVLHTDHCAK.K (111)	C12-Bimane C17-CAM	1.34 ±0.28
8	Fructose-1,6-bisphosphate aldolase	FG02770.1	67% (12) 2150	K.VNVDTDMQFAYCSGIR.D (90) R.SLAPTYGIPVVLHTDHCAK.K (103)	C12-Bimane C17-CAM	0.76 ±0.19
9	Phosphoglycerate kinase	FG03992.1	87% (25) 13879	K.VTFAPDCVGPEVEEIVNK.A (109) K.DANTGYATDKDGIPDGWQGLDCGEESVK.L (129) K.VNTLIICGGMAFTFK.K (91) K.DGIPDGWQGLDCGEESVK.L (98)	C7-Bimane C22-Bimane C7-CAM C12-CAM	0.70 ±0.16
10	Glyceraldehyde 3-phosphate dehydrogenase	FG06257.1	86% (16) 18127	K.YDGSADIISNASCTTNCLAPLAK.V (110)	C13-Bimane, C17-CAM	0.31 ±0.02
13	Protein similar to mannitol dehydrogenase	FG04826.1	66% (8) 5626	K.AAYVYFLSDASTYTTGSDLVIDGGYTCR.- (125)	C27-Bimane	1.90 ±0.56
14	Protein similar to mannitol dehydrogenase	FG04826.1	65% (9) 3285	K.AAYVYFLSDASTYTTGSDLVIDGGYTCR.- (123)	C27-Bimane	1.50 ±0.12
15	Cytochrome c oxidase subunit 4	FG02712.1	25% (5) 359	R.SAGEEQFAGCTGFPADSHSVTWLGITR.E (78)	C10-Bimane	1.93 ±0.28
	Dihydrodiol dehydrogenase (NADP)	FG11142.1	6% (1) 37	K.NCLDAIAAG.H (37)	C2-Bimane	
16	Cytochrome c oxidase subunit 4	FG02712.1	27% (6) 624	R.SAGEEQFAGCTGFPADSHSVTWLGITR.E (116)	C10-Bimane	1.57 ±0.33
18	Alkyl hydroperoxide reductase 1 (Sce)	FG08677.1	82% (6) 701	K.VVLISVPGAFTPTCSASHVPSYVENIEQIK.A (22)	C14-Bimane	2.22 ±0.65

Spot[1)]	Putative identity[2)]	ID[3)]	P/C[4)] Score[5)]	Cysteine peptides[6)] Ion Score[7)]	Modified Cys[8]	Flo:Total Protein[9)] Mean±Std Dev
19	Peroxiredoxin 5	FG00353.1	68% (7) 1707	K.TSNGYIVGVPAAFSGTCSSK.H (31)	C17-Bimane	2.62 ±0.49
20	40S ribosomal protein S12	FG07292.1	83% (8) 437	R.KVVNCSCVVVK.D (59) K.QLGEWAGLCVLDR.E (27)	C5-CAM, C7-Bimane C9-Bimane	1.3 ±0.34
23	Probable brt1 protein	FG00609.1	72% (5) 892	K.TPHMIYCSGQIPLTPEGELVQGITEQTR.Q (111)	C7-Bimane	1.75 ±0.13

All spectra matched proteins in *F. graminearum* genome and cysteine modification sites were tentatively assigned.

1) Spots with differential mBBr signals, excised from 2-D gels stained with Coomassie Blue (Fig. 4.3).

2) Putative identity obtained by BLASTP search of putative *F. graminearum* protein versus NCBInr.

3) Accession numbers for the respective proteins according to the *F. graminearum* genome database

4) Percentage coverage and number of statistically significant matched peptides in parenthesis; including missed cleavage

5) MASCOT total ion score for protein.

6) Peptide sequence containing modified cysteine; CAM: Iodoacetamide modification (+57Da); Bimane: mBBr modification (+190Da)

7) The highest observed MASCOT ion score for the peptide; higher than 33 indicate identity or extensive homology ($p < 0.05$); in parenthesis

8) Assigned site of cystiene modification

9) Mean Ratio of Florescence to Total protein content for the corresponding spot

Also, MS analysis of protein spots extracted from 2D gels often leads to identification of multiple proteins from the same spot. This is due to overlapping of proteins with very close pI values or a shift in pI caused by post-translational modifications or amino acid change (isoforms). In our study, Spot # 15 contained at least two proteins namely Cytochrome c oxidase subunit 4 (FG02712.1) and Dihydrodiol dehydrogenase (NADP dependent) (FG11142.1), both containing bimane labeled cysteines.

Selective labeling of reduced disulfides by mBBr provides a covalent bimane modification (Bimane; mass shift of +197.074 Da) while free thiols blocked by IAM get carbamidomethylation (CAM; mass shift of +57.034 Da) as compared to native free –SH group of cysteine (Figure 4.6). CID fragmentation patterns showed that the covalent bond between mBBr and the thiol group remains intact during fragmentation. MS analyses of these modifications allowed us to distinguish peptides containing redox responsive cysteines from those containing free thiols as well as precise mapping of reactive cysteines. In total, 12 reactive (Cys-bimane) and 5 non-reactive (Cys-CAM) sites of cysteine modification localized in 15 peptides from 10 proteins were identified. Out of them, 10 unique peptides showed only bimane modified cysteines. 3 unique peptides were obtained with free native thiols showing CAM modification. In 2 unique peptides both bimane and CAM modifications were observed. Interestingly, Phosphoglycerate kinase (FG03992.1) was found to have 4 cysteines in 4 unique peptides; 2 reactive whereas other 2 non-reactive thiols. It is a very good example of the presence of distinct reactive and non-reactive thiols to Nox activity within a single protein. Presence of both Cys-bimane and Cys-CAM in the same peptides or peptides within the same protein

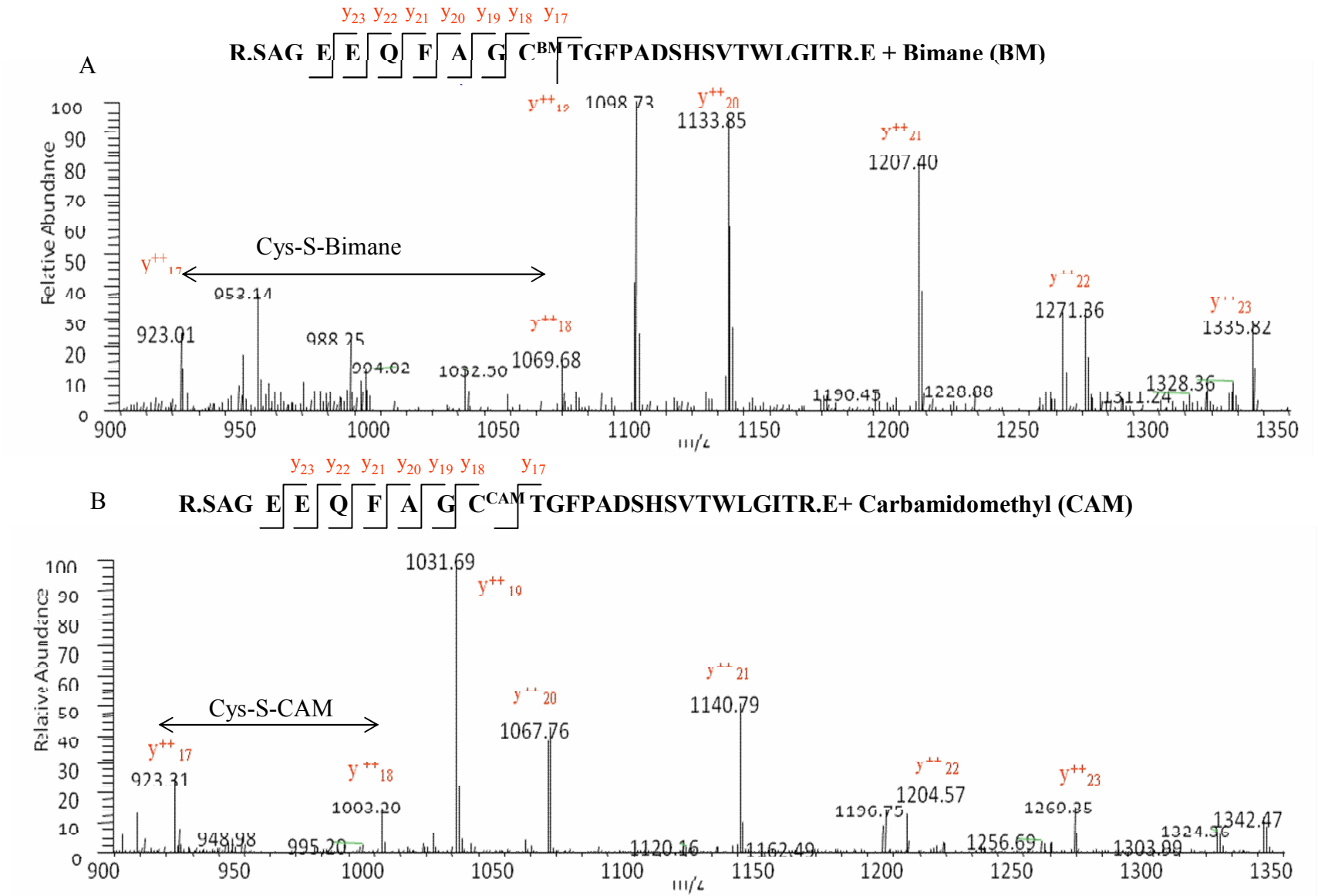


Figure 4.6 Detection of differentially labeled cysteine in peptide R.SAGEEQFAGCTGFPADSHSVTWLGITR.E representing Cytochrome c oxidase subunit 4 (FG02712.1) at spot number 16 (Table 4.1). Fragmentation spectra of peptide ions with Cys-bimane, precursor m/z 1005.96, in WT (A) and Cys-CAM, precursor ion m/z 961.62, in Δ*noxAB* protein extracts. Sequence specific y^{++} ions and delta mass difference between y^{++}_{17} and y^{++}_{18} ions corresponding to bimane and CAM modifications are indicated.

increased the confidence in the assignment of modification among the reactive and non-reactive thiols.

4.2 Shot Gun proteomics:

4.2.1 Biotin enrichment of reversibly oxidised cysteines

To examine the redox proteins in more detail and with improved coverage, gel-free enrichment of redox peptides followed by MS analysis was conducted. Affinity of biotin towards streptavidin enabled us to enrich the peptides containing reactive thiols now tagged with biotin-HPDP. Peptides with alkylated free thiols and/or no cysteines were unable to bind to the affinity column and were washed out. Elution using β-mercaptoethanol removed biotin tag and modified the reactive thiols by adding $–OC_2H_5$ (+76 Da) to the native cysteine. This facilitates mass spectrometric identification of peptides containing redox responsive cysteines to Nox activity (Figure 4.7). The eluted peptides forming the 'redox peptidome' were subjected to LC-MS/MS analysis.

4.2.2 Identification of Nox targeted reversibly oxidised proteins

In this study, the complexity of proteomes is significantly decreased as we isolated the peptides containing reactive thiols. Comparison of redox active peptides between Nox deletion mutant and wild type further narrows down the identification of Nox- targeted proteins among the global redox responsive proteins in *F. graminearum*. A total of 96 proteins containing reversibly oxidised cysteine(s) were found altogether in WT and Δ*noxAB* at least in one replicate. Out of 96, 29 proteins were consistently present in all three wild type replicates. Only proteins with ion score >95% were taken. Manual inspection of MS/MS spectra was carried out to confirm that assignment of cysteine oxidation in target proteins was correct and identification was not based solely on

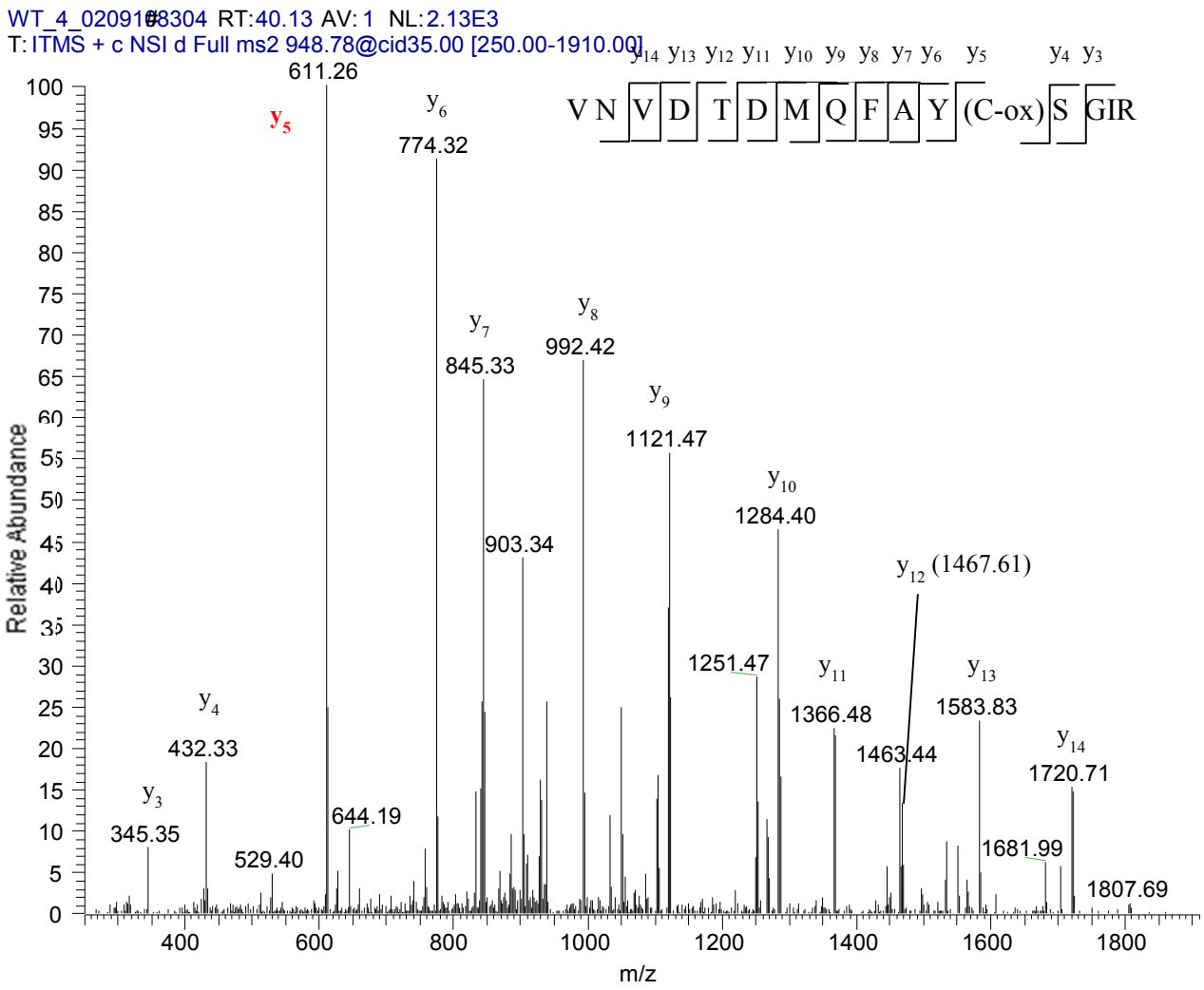


Figure 4.7 MS/MS spectrum of peptide VNVDTDMQFAY(C-ox) SGIR representing Fructose-1, 6-bisphosphate aldolase (FG02770.1) (Table 4.2). Precursor ion $[M+2H]^{2+}$ (m/z = 948.78) yielded singly charged y-ion series as labeled. The theoretical mass and observed mass of diagnostic y^5-ion, shown in red, are 535.24 Da and 611.26 Da respectively; indicating a deviation of 76.02 Da consistent with β-mercaptoethanol modification at cysteine. The y-ion series labeled in the spectrum is marked on the peptide sequence shown above.

MASCOT score and E value. The identified candidate proteins are listed in Table 4.2 together with the peptide containing oxidised cysteine(s), assigned site of cysteine modification and the highest MASCOT score for the individual ion. From 29 proteins, 37 unique peptides containing 39 reversibly oxidised cysteine(s) (destreak modification) and 2 free thiols (CAM modification) were found. 22 proteins contain a single peptide while seven proteins contain two or more peptides with modified cysteine(s). Among these seven proteins, pyruvate decarboxylase (FG09834.1) contains four peptides and 60S ribosomal protein L4-A (FG07186.1) contains three peptides, with one reactive cysteine in each peptide. Two proteins, protein disulfide-isomerase (FG01246.1) and alcohol dehydrogenase 1 (FG10855.1), were found to contain differentially modified cysteines within the same peptide with oxidised thiols at C_{17} and C_5 and free native thiols at C_{14} and C_{15} respectively. Presence of both modified forms at different cysteines increased the confidence in assignment of the reactive cysteine modification site. An uncharacterized protein FG05175.1 was shown to contain multiple reactive cysteines (C_2, C_7 and C_{23}) within the same peptide. Detection of the same site of cysteine modification with high ion score for a corresponding protein in all three WT replicates increased the confidence in identification of candidate Nox target proteins. In case of low score, MS/MS fragmentation was manually inspected and only credible matches were accepted.

Four candidate proteins fructose-1, 6-bisphosphate aldolase, cytochrome c oxidase subunit 4, phosphoglycerate kinase and mannitol dehydrogenase were identified in both gel and gel-free experiments. The peptides containing reversibly oxidised cysteines in these proteins were found to be the same using both approaches.

Table 4.2 **Identification of reversibly modified proteins in *F. graminearum* wild-type and Δ*noxAB* , by LC-ESI-MS/MS and MASCOT, using non-gel approach**

#	ID[1)]	Putative Protein ID[2)]	Significant Peptide[3)]	Ion Score[4)]	Site of Cys-Ox[5)]
1	FG 00048.1	Hypothetical protein (Fgr)	K.DLVPDQVATTEAETFFSGLFNNCIGDNIFVNHLTTYPR.V	115	C 23
			R.LLEVITGGVCVATTHR.V	106	C 10
2	FG 00049.1	Related-to-branched-chain-amino-acid-aminotransferase (Aor)	R.KLSTSLDDIMHCR.V	51	C 12
3	FG 00421.1	S-adenosylmethionine synthetase (Ncr)	K.IADQVSDAILDACLR.E	121	C 13
			K.TCNLLVAIEQQSPDIAQGLHYEK.A	123	C 2
4	FG 00802.1	60S ribosomal protein L23 (Sce)	K.MTLGLPVGAVMNCADNSGAR.N	130	C 13
5	FG 00838.1	Heat shock 70 kDa protein (Ncr)	R.FEELCQDLFR.S	72	C 5
6	FG 01246.1	Protein disulfide-isomerase (Hin)	K.SNDLVLAEFFAPWCGHCK.A C 14: Carbamidomethyl, C17: Destreak	64	C 17
7	FG 01425.1	H+ ATPase (Sce)	K.LSAIESLAGVEILCSDK.T	45	C 14
8	FG 02712.1	Cytochrome c oxidase subunit 4 (Ncr)	R.SAGEEQFAGCTGFPADSHSVTWLGITR.E	141	C 10
9	FG 02770.1	Fructose-1,6-bisphosphate aldolase (Aor)	K.VNVDTDMQFAYCSGIR.D	140	C 12
10	FG 03535.1	Trichodiene oxygenase (tri4) (Fgr)	R.MIAPSVCDLLQMR.D	85	C 7
11	FG 03992.1	Phosphoglycerate kinase (Fgr)	K.KVTFAPDCVGPEVEEIVNK.A	74	C 8
			K.DANTGYATDKDGIPDGWQGLDCGEESVK.L	132	C 22
12	FG 04558.1	Carbonic anhydrase (Ani)	R.IPAEQICGLEPGEAFIHR.N	85	C 7
13	FG 04826.1	Similar to mannitol dehydrogenase (Fgr)	K.AAYVYFLSDASTYTTGSDLVIDGGYTCR.-	58	C 27
14	FG 05175.1	Uncharacterized protein	R.DCSAAICNSEQAAAVVAYGLALCR.Q	129	C 2, C 7, C 23
15	FG 06019.1	60S ribosomal protein S5 (Fgr)	K.SIAECLAEELINAAK.G	121	C 5
16	FG 06847.1	14-3-3 protein homolog (Fgr)	K.ICEDILEVLDQHLIPSAK.S	99	C 2
17	FG 07186.1	60S ribosomal protein L4-A (Spo)	R.AGQAAFGNMCR.S	60	C 10
			R.YAVCSALAASAAVPLLQAR.G	78	C 4
			R.NITGVETCPVTALNLLQLAPGGHLGR.F	65	C 8

#	ID[1)]	Putative Protein ID[2)]	Significant Peptide[3)]	Ion Score	Site of Cys-Ox[5)]
18	FG 07361.1	Probable succinate dehydrogenase (Spo)	R.NLLTCAIQTAESAANRK.E R.AAFGLAEAGFNTACISK.L	100 92	C 5 C 14
19	FG 07528.1	Pyruvate kinase (Fgr)	K.RPVSTVESCAMAAVR.A	71	C 9
20	FG 08077.1	Putative NADPH dehydrogenase (Spo)	R.LFLAPLCQYSAK.D	64	C 7
21	FG 09321.1	Probable-acetoacetyl-CoA-thiolase (Ncr)	K.DHMGISAELCVDDHDLTR.E K.ILGLNPDQVNVYGGSVAIGHPLGCSGAR.I	102 110	C 10 C 24
22	FG 09574.1	Elongation factor 2 (Ncr)	R.VTDGALVVVDTVEGVCVQTETVLR.Q	74	C 16
23	FG 10089.1	Related-to-sporulation-specific-gene-SPS2 (Afu)	K.LDQVSGSVVVSSTTDIEEFCK.Y	139	C 20
24	FG 10118.1	Ketol-acid reductoisomerase (Ncr)	K.VEVPTDIDVILCAPK.G	113	C 12
25	FG 10246.1	Similar to ribosomal L10 protein (Fgr)	R.ANVDDFPLCIHLVSNEYEQLSSEALEAAR.I	111	C 9
26	FG 10264.1	Glutamine synthetase (Fgr)	R.GSPNIIVLAECYDSDGTPNK.Y	137	C 11
27	FG 10737.1	Probable Adenylate kinase (Fgr)	K.ECQGGFILDGFPR.T	97	C 2
28	FG 10855.1	Alcohol dehydrogenase 1 (Ncr)	K.FSGVCHTDLHAWQGDWPLDTK.L R.IPEECDLEAISPILCAGITVYK.G C 15: Carbamidomethyl	73 90	C 5 C 5
29	FG 10905.1	40S ribosomal protein (Fso)	M.APSNLPSVMNATSQDIETLLAAQCHLGSK.N	100	C 24

All spectra matched proteins in *F. graminearum* genome and cysteine modification sites were tentatively assigned.

1) Accession numbers for the respective proteins according to the *F. graminearum* genome database

2) Putative identity obtained by BLASTP search of putative *F. graminearum* protein versus NCBInr (January 2008).

3) Peptide sequence containing modified cystiene; CAM:Iodoacetamide modification (+57Da); Destreak:β-mercaptoethanol modification (+76Da)

4) The highest observed MASCOT ion score for the peptide [29].

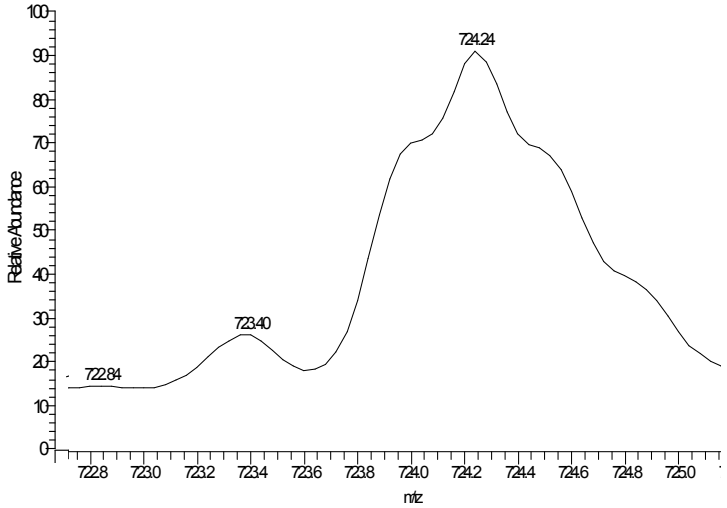
5) Assigned site of reversibly oxidised cystiene

Afu: *Aspergillus fumigatus*
Ani: *Aspergillus niger*
Aor: *Aspergillus oryzae*
Ate: *Aspergillus terreus*
Fso: *Fusarium solani*
Hin: *Humicola insolens*
Ncr: *Neurospora crassa*
Sce: *Saccharomyces cerevisiae*
Spo: *Schizosaccharomyces pombe*

4.2.3 Confirmation of Cys-Ox state

To confirm that the identified proteins in WT are the candidate targets of Nox enzymes, it was important to confirm the thiol status in corresponding proteins in Δ*noxAB*. Nox activity increases the ROS levels in wild-type cells, shifting the cellular redox status towards more oxidised, resulting in oxidised form of thiols (Cys-Ox) in target proteins as opposed to the native reduced state of the corresponding thiols (Cys-Red) in Δ*noxAB* proteins. Peptides representing the target proteins in WT were able to adhere to streptavidin column due to their cysteines tagged with biotin, but not in Δ*noxAB* because the reduced thiols were blocked by IAM. The flow through, post incubation of peptides with streptavidin, of both WT and mutant samples were analyzed by MS/MS analysis. A comparative analysis of cysteine state in significant peptides for 29 target proteins was done by studying WT affinity elute (AE) and flow through (FT) against Δ*noxAB* AE and FT. Of the 29 target proteins, 22 proteins showing Cys-Ox state in WT AE (showing destreak modification; and not found in Δ*noxAB* AE) were found to have Cys-Red state in Δ*noxAB* FT (blocked with IAM, thus showing Cys-CAM modification). Specific peptides for 7 proteins (Cys-Ox state in WT AE; and not found in Δ*noxAB* AE) were either found in unmodified Cys-SH state or were not found in Δ*noxAB* FT. For these 7 proteins SIM scan mode was employed to detect the relevant precursor ion in Δ*noxAB* AE, utilizing elution times known from previous MS/MS analysis of WT sample; some minor retention time shifting (~0.3 min) was kept in consideration. The chromatographic scans for these precursor ions are shown in Figure 4.8. Signals were recorded at selected m/z (Figure 4.8 A, C, E, G, I, K and M) in affinity elute of WT but were not detected or observed at significantly low intensity in that of Δ*noxAB* (Figure 4.8

A.


Relative Abundance
m/z
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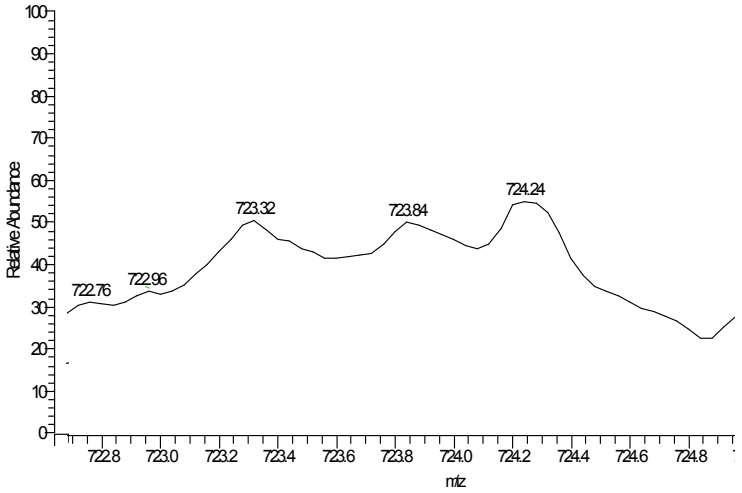
723.40
724.24

B.


Relative Abundance
m/z
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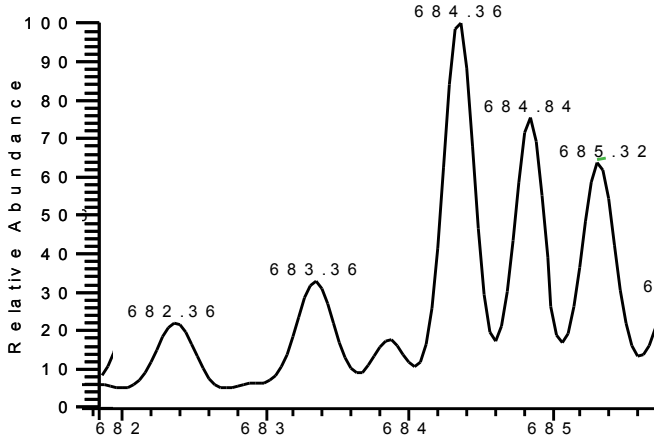
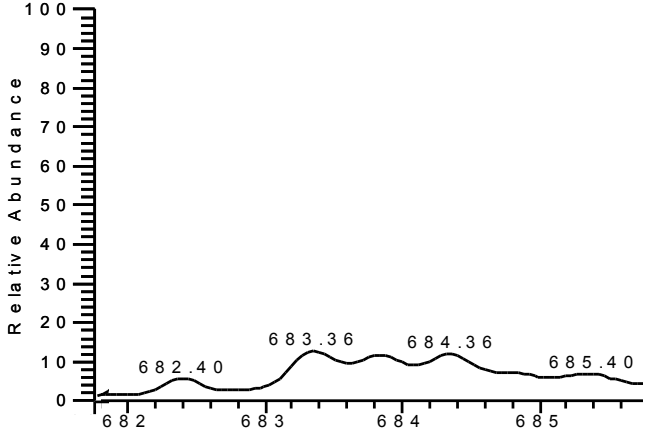
722.96
723.32
723.84
724.24

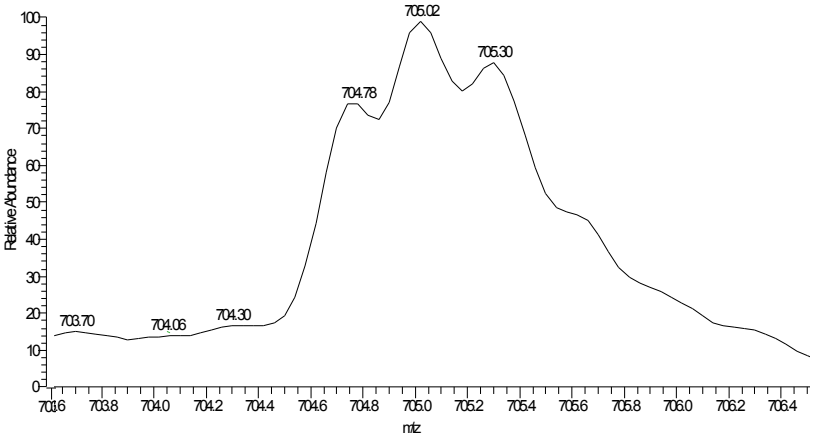
C


Relative Abundance
682.36
683.36
684.36
684.84
685.32

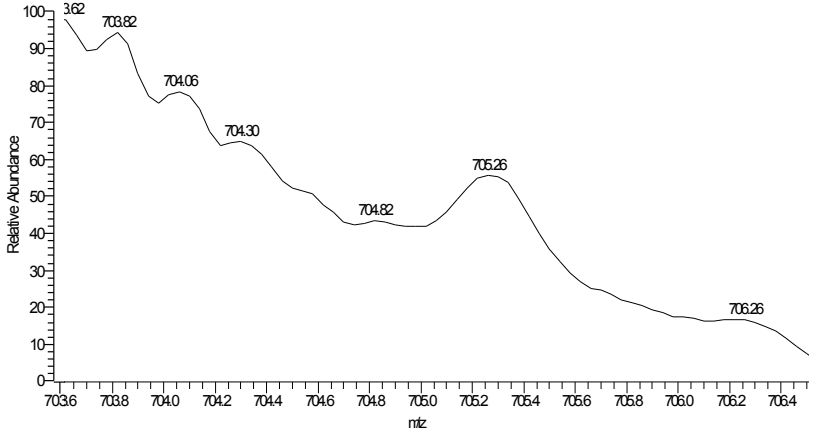
D


Relative Abundance
682.40
683.36
684.36
685.40

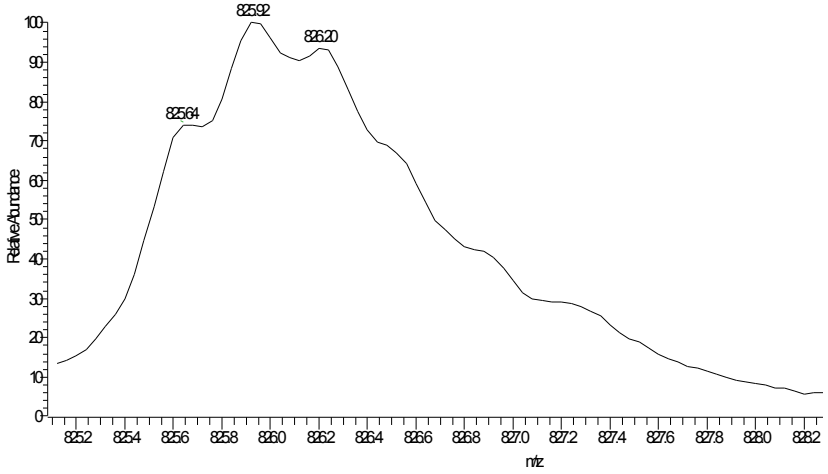
E


Relative Abundance
m/z
703.70
704.06
704.30
704.78
705.02
705.30

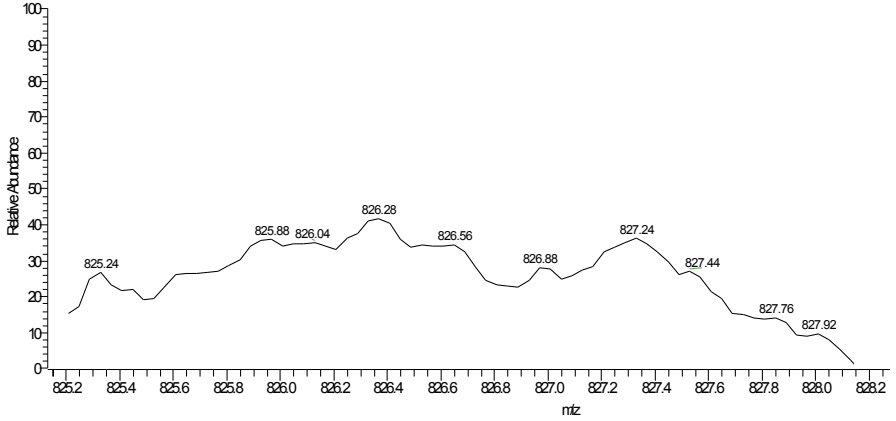
F


Relative Abundance
m/z
703.62
703.82
704.06
704.30
704.82
705.26
706.26

G


Relative Abundance
m/z
825.64
825.92
826.20

H


Relative Abundance
m/z
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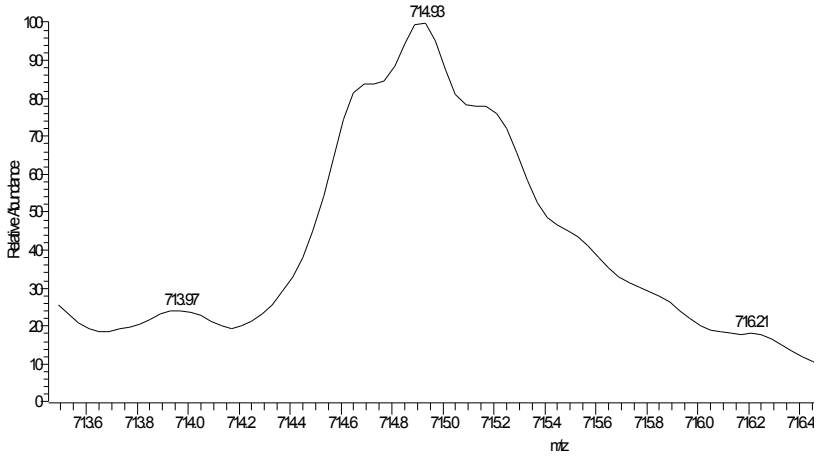
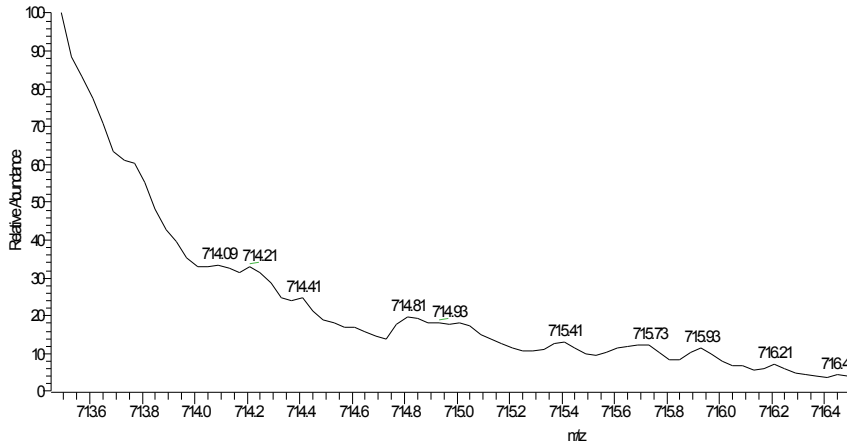
825.88
826.04
826.28
826.56
826.88
827.24
827.44
827.76
827.92

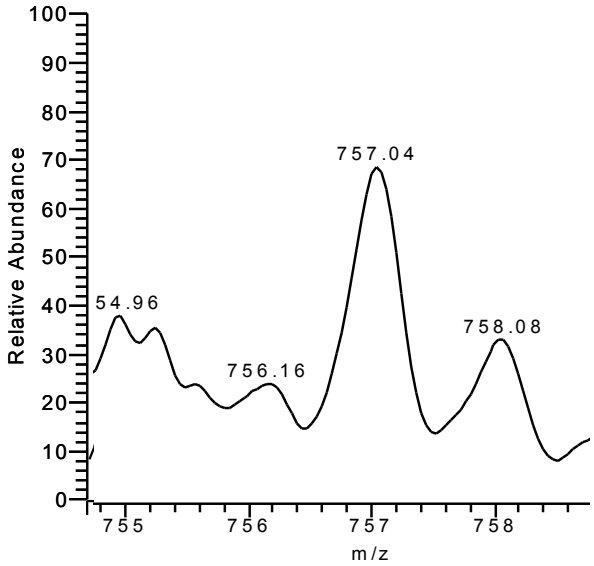
I


714.93
713.97
716.21
Relative Abundance
m/z

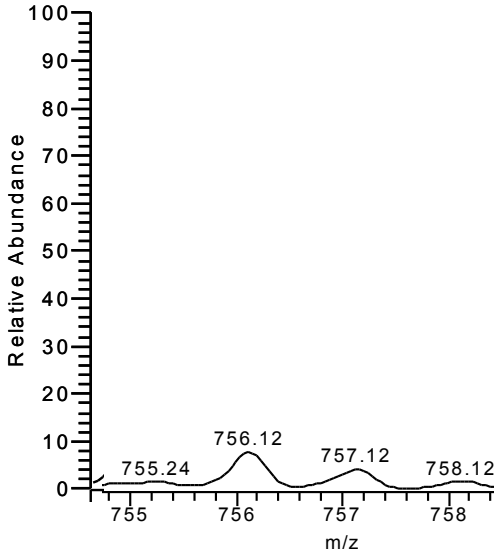
J


714.09
714.21
714.41
714.81
714.93
715.41
715.73
715.93
716.21
Relative Abundance
m/z

K


757.04
754.96
756.16
758.08
Relative Abundance
m/z

L


756.12
755.24
757.12
758.12
Relative Abundance
m/z

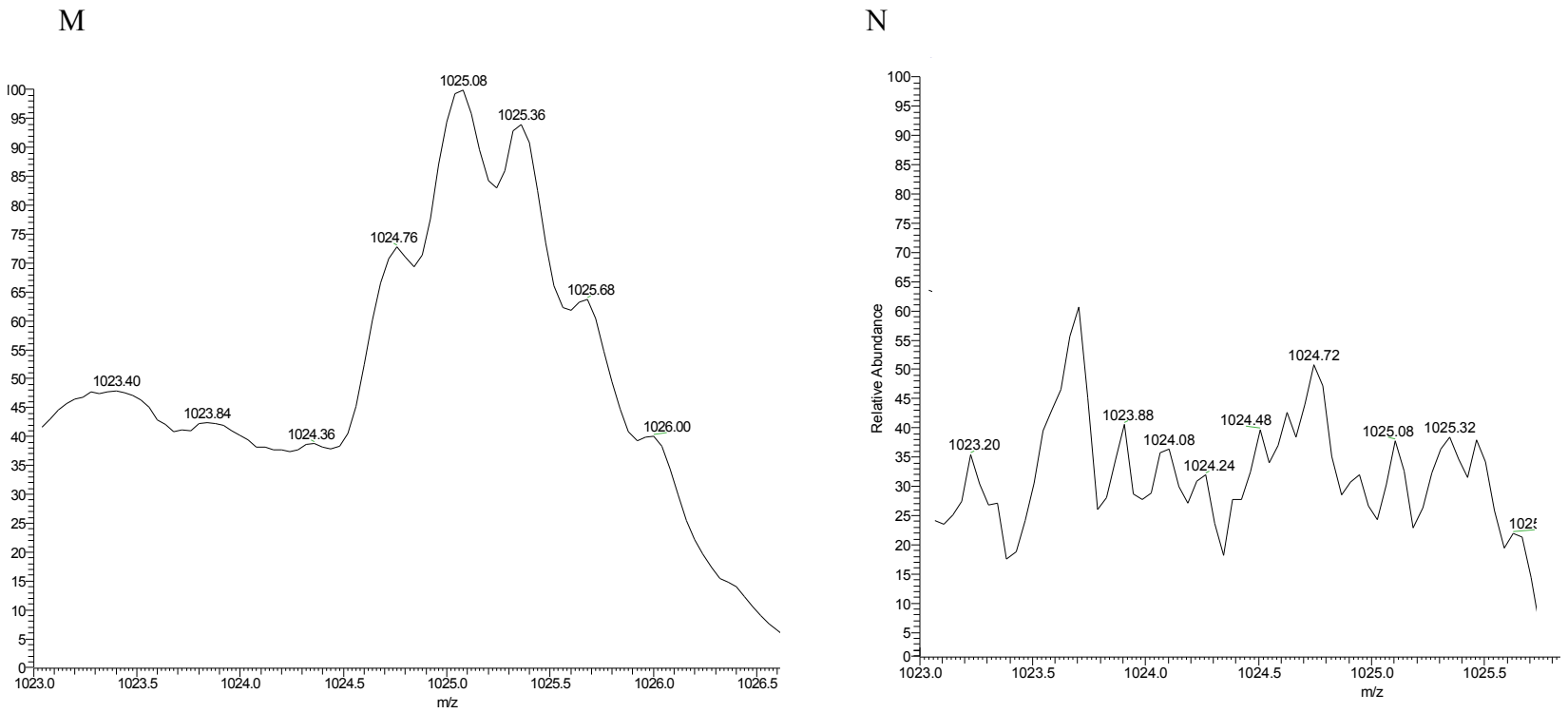


Figure 4.7 Selected Ion monitoring (SIM) chromatograms of diagnostic ions in biotin-affinity elute of WT (A, C, E, g, I, K and M) and Δ*noxAB* (B, D, F, H, J, L and N) (Table 3.1). Precursor ions for Protein disulfide-isomerase: m/z 724.20, 3+ (A and B); Carbonic anhydrase: m/z 684.31, 2+ (C and D); 14-3-3 protein homolog: m/z 704.98, 3+ (E and F); Pyruvate kinase: m/z 826.45, 3+ (G and H); Putative NADPH dehydrogenase: m/z 715.03, 3+ (I and J); Adenylate kinase: m/z 757.05, 2+ (K and L); and 40S ribosomal unit: m/z 1025.10, 3+ (M and N) showing presence and absence in elute of WT and Δ*noxAB* respectively.

B, D, F, H, J, L and N) thus demonstrating that the Cys-Ox state in the selected peptides in WT was unlikely to be present in case of mutant proteins. MS/MS spectra of these were acquired too and compared manually to MS/MS spectra of the peptide in original WT sample.

4.3 Functional distribution of candidate oxidised proteins

Data obtained from both proteomic approaches showed that most of the Nox targets are enzymes, primarily involved in metabolism, oxidation-reduction processes and cellular thiol homeostasis. A pie chart distribution of candidate proteins based on the biological processes they are involved in is shown in Figure 4.9. A total of 35 candidate proteins included 12 metabolic enzymes (34%), 7 enzymes involved in electron transfer reactions (20%) and 3 thiol-oxidoreductases involved in antioxidant systems (9%). One elongation factor (EF-2) involved in protein synthesis (3%) and 6 ribosomal units involved in ribosome biogenesis (17%) were identified. Among others were one protein (Protein ecm33) involved in sporulation and conidial/mycelial cell wall biosynthesis, one 14-3-3 homolog protein with role in fungal filamentous growth and pathogenicity, one heat shock protein (Hsp70) and 3 proteins with unknown functions in *F. graminearum*.

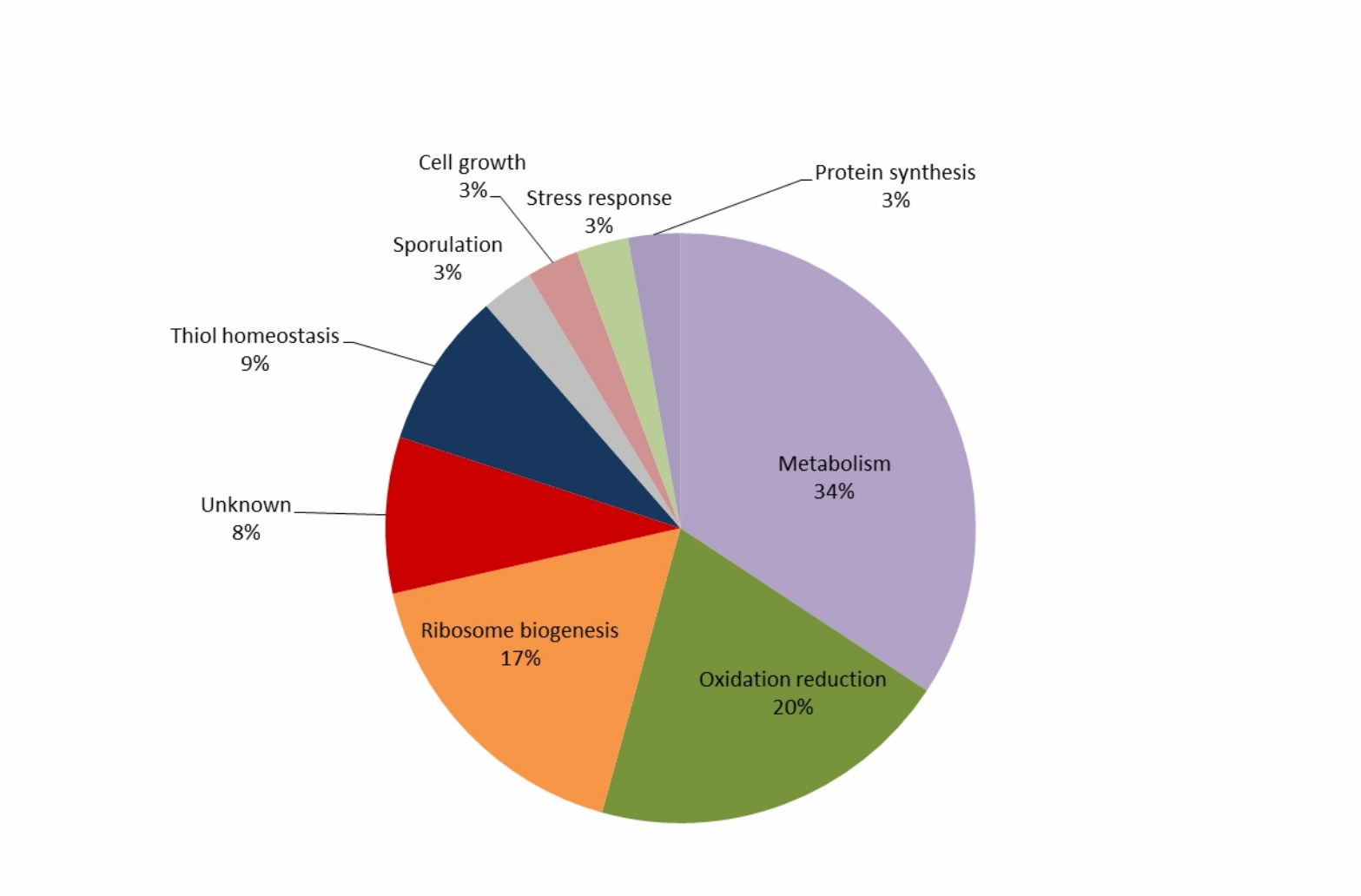


Figure 4.9 Functional distributions of identified reversibly oxidised proteins

DISCUSSION

Chapter 5: Discussion

In an effort to identify Nox targets expressed during pathogenesis, *F. graminearum* WT and Δ *noxAB* strains were grown in nitrogen limited, tricothecene-inducing conditions. Nitrogen limitation is a known cue that regulates fungal gene expression during infection. Tricothecenes are secreted concurrently to the beginning of infection process, thus proteins contributing to pathogenesis are expressed under conditions favouring toxin production (Taylor et al., 2008). Nutritional stress triggers the activity of Nox enzymes (Jiang, 2011) thus generating differential WT and Δ *noxAB* proteomes, in terms of post translational cysteine oxidation. 2D gel and gel-free based redox proteomics approaches were employed for their comparative study. We identified 35 total proteins as potential Nox targets, 10 from 2D gel and 29 from gel-free systems with 4 common proteins in both experiments. A few of the candidate proteins have been identified earlier as pathogenicity proteins in overall proteomic analyses of *F. graminearum* grown under DON-inducing conditions (Taylor et al., 2008). Also, there is a significant degree of overlap between the oxidised proteins identified in our study and the previous studies done in yeast, plants, animals and human plasma (McDonagh et al., 2009; LeMoan et al., 2006; Lee et al., 2004; Mirzaei et al., 2008; Fratelli et al., 2002). This supports the use of Cys-targeted proteomics as a robust method for investigating redox proteins. Several proteins identified in this study are known targets of thioredoxins and/or glutathionylation. This supports the hypothesis that these candidate proteins are involved in reversible thiol modification. Yet these studies involved global oxidizing conditions and no reports on fungal Nox targets have been published so far. To the best

of our knowledge, this is the first report on Nox targeted redox disulfide proteome of *F. graminearum*.

5.1 *F. graminearum* disulfide proteome: A comparative analysis

Differences observed between WT and Δ*noxAB* total proteomes were minor as PTMs including cysteine oxidation modulate protein functions/localization without significant quantitative differences (Kim et al., 2006; Moon et al., 2006). As anticipated WT redox disulfide profile showed higher oxidation levels, visually evident as higher mBBr signals, due to Nox activity. Considering that cysteine oxidation is a subtle, dynamic redox change and few proteins contain cysteine residues, the redox disulfide proteome formed a small subset of total proteome. Thiol-modification due to ROS is very sensitive to pH (*in vitro*) and some loss due to incomplete alkylation and reduction reactions can also occur. The gel-free system resulted in a higher coverage of the redox proteome than 2DE, including proteins with increased hydrophobicity and low abundance. 2D gel was used for a global comparison of thiol status and overall protein profiles. Also, intact proteins from gels were excised, trypsin digested and analysed in MS; thus information about total number of cysteine residues, presence of free thiols localized in other peptides and isoforms of a protein was obtained by 2DE.

Our study aimed for a specific subset of the *F. graminearum* redox-sensitive proteins which are oxidised due to the contribution of Nox enzymes in increasing the cellular ROS concentrations. Thus a comparative analysis of redox proteomes of Nox deletion mutants with wild-type enabled to distinguish proteins specifically oxidised due to Nox activity than proteins affected by global changes in ROS levels. Three groups of proteins with differential redox response (thiol state) and abundance in the total proteome

were observed in this study. Firstly, the proteins containing reversibly oxidised cysteines only in WT yet present in both WT and mutant total proteomes; representing the targets of Nox enzyme activity. Secondly, the proteins with oxidised thiols in both WT and mutant redox proteomes which represent either constitutively oxidised proteins or redox responsive proteins but non-targets of Nox enzyme. Thirdly, the total proteome minus redox proteome, i.e. the pool of proteins which either does not contain reversibly oxidised thiols or any cysteine residues. Also in 2D gels, the proteins fluorescing with high intensity but very faint in Coomassie stained gels represented low abundant redox responsive proteins.

5.2 Nox mediated redox responding proteins

Fungal pathogenicity is a cumulative response to a multitude of biological and environmental changes. During early stages of infection, central carbon metabolism and nitrogen assimilation pathways in a fungus undergo changes other than normal growth state and cellular redox homeostasis is inclined towards higher oxidizing levels. Most of the identified Nox targets play role in primary metabolism and thiol-redox reactions, thus underscoring the role of redox signalling during the induction of pathogenicity. In the following, implications of reversible modifications of these proteins and the pathways they are involved in regarding to fungal virulence related changes are discussed.

5.2.1 *F. graminearum* metabolic pathways

Glycolytic enzymes such as FG06257.1 Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), FG03992.1 Phosphoglycerate kinase (PGK), FG07528.1 Pyruvate kinase (PK) and FG02770.1 Fructose bisphosphate aldolase (FBA) were identified as Nox targeted proteins. GAPDH, PGK and PK enzymes are reversibly

inhibited in response to thiol oxidation (McDonagh et al, 2009; LeMoan et al., 2006, Shenton and Grant 2003; Fratelli et al., 2002). Switching off their enzymatic activities could be a regulated way to redirect energy from glycolysis to the pentose phosphate pathway (PPP). It is known that a subgroup of glycolytic and TCA cycle enzymes is inhibited, while PPP activity is enhanced under increased oxidative conditions (Godon et al., 1998, Ralser et al., 2007). NADPH depletion by Nox enzymes is balanced by NADPH production via PPP in response to ROS sensing. NADPH also serves as reducing power for anti-oxidant system in order to control the levels of cellular ROS. GAPDH was shown to contain two cysteine residues Cys^{152} and Cys^{156} localized in a single tryptic peptide, and Cys^{152} was detected as reversibly oxidised thiol. It is reported that the reactive cysteine in GAPDH is a major target of S-thiolation, a reaction by which protein thiols form mixed disulphides with glutathione, and thus reversibly inactivates it (Shenton and Grant 2003). Two reactive cysteines were detected in PGK at Cys^{99} and Cys^{316}. The catalytic properties of PGK are known to be sensitive to structural modification (Ritco-Vonsovici et al., 1995). Allosteric regulation of PK involves a cysteine residue at binding site for its activator Fructose-1, 6-bisphosphate, which was reported to be reversibly oxidised by glutathione (McDonagh et al., 2009). A reactive, surface cysteine residue in FBA has been found to undergo S-thiolation however oxidation does not affect its activity (Shenton and Grant 2003). This could be explained because inactivation of aldolase would prevent recycling of PPP generated ribose-5-phosphate to glucose-6-phosphate for subsequent NADPH production. FBA is involved in both gluconeogenesis and glycolysis, and is essential for growth and virulence of *C. albicans* and thus represents a potential antifungal target (Rodaki et al., 2006).

FG07361.1 is a probable succinate dehydrogenase (SDH), shown to contain two reversibly modified thiols Cys^{82} and Cys^{568} localized in two peptides. Though cysteine modification is dispensable for succinate oxidation, it is likely to have a role in structural stability of the enzyme as shown by enzymatic inhibition on treatment with thiol-specific reagents (Hederstedt and Hedén 1989; Lemire and Oyedotun 2002). FG10855.1 Alcohol dehydrogenase (ADH) was found to contain three cysteines in two peptides; Cys^{82} and Cys^{192} were reactive whereas Cys^{182} was non-reactive. Yeast ADH has been reported to be inactivated by H_2O_2, and restored by thiol-reducing agents, implying a reversible modification (Men and Wang 2007). Reversible inhibition of ADH would result in non-regeneration of NAD^+ and slowing down of glycolysis.

Fungal glycolysis and TCA cycle declines in the early stages of infection when availability of glucose may be low, but resumes afterwards during host tissue colonization (Barelle et al., 2006). Histochemical studies on *M. grisea* and temporal analysis in *Tapesia yalundae* suggested that lipid catabolism provides an essential carbon source throughout the germination and penetration phases (Thines et al., 2000; Weber et al., 2001; Bowyer et al., 2000). Breakdown of lipid provides the glycerol required to generate turgor pressure in the appressoria in *M. grisea* (de Jong et al., 1997). Keeping this in view, our findings advocate for Nox mediated redox signals regulating the metabolic changes in the pathogen when the fungus prepares for infection.

A very important metabolic enzyme FG10264.1 Glutamine synthetase (GS) was identified to undergo Nox mediated reversible thiol modification in our study. GS is essential in nitrogen metabolism of filamentous fungi to form glutamine. The intracellular glutamine levels define the N-status of the cell and regulate activation and/or suppression

of several processes that require dissipation of energy (Marzluf 1997). Nitrogen limitation is a cue for expression of virulence proteins, so far shown in vitro, and redox repression of GS might be involved in signalling the non-availability of resources. Relation between glutamine and the target of rapamycin (Tor) kinase signalling pathway are well documented (Komeili et al., 2000; Crespo et al., 2002). Recently *F. oxysporum* virulence-related proteins were shown to express after treatment with methionine sulfoximine or rapamycin, two specific inhibitors of GS and Tor kinase, respectively (López-Berges et al., 2010). Tor pathway perceives the nutrition information, in particular nitrogen, and regulates cell growth and differentiation (Rohde et al., 2004), as well as protein synthesis (Nakashima et al., 2008). Glutamine also acts as a precursor of glutamate, which is likewise required for synthesis of the antioxidant glutathione. Redox mediated inhibition of GS would also be a route to keep GSH levels low in order to maintain the oxidizing status of cellular redox required during infection (Matés et al., 2002).

Gene deletion studies done on the methionine synthase gene *msy1* and cystathionine β-lyase *cbl1* suggest that methionine pathway is critical for plant infection in *F. graminearum* (Seong et al., 2005). We identified FG00421.1 S-adenosylmethionine synthetase (SAM synthetase) as a Nox target for oxidation. It is involved in cysteine and methionine metabolism, and biosynthesis of secondary metabolites in fungi. There are 4 cysteines in its amino acid sequence, among which two Cys^{51} and Cys^{110} were detected as reversibly oxidised. SAM synthetase catalyzes formation of S-adenosylmethionine (SAM) from methionine, which allosterically activates cystathionine β-synthase (CBS), the principal enzyme in the trans-sulfuration pathway. Methylation and antioxidant

metabolism are linked by the trans-sulfuration pathway, which changes the methionine cycle intermediate homocysteine to cysteine, the limiting agent in glutathione (GSH) synthesis. Prudova et al (2006) explored that SAM synthetase activity modulates redox capacity in terms of GSH, since SAM dependent inactivation of CBS led to declined GSH and increased oxidation levels in the cell. Interestingly –SH groups control activity and/or structure of SAM synthetase (Martínez-Chantar and Pajares, 1996) through the formation of intramolecular disulfides. This is in agreement with our finding of two reversibly modified cysteines in the enzyme. Development of sexual cleistothecia (reproduction/fruiting bodies) requires low GSH: GSSG levels, high GSH levels ensue in the development of asexual conidiophores (Thon et al., 2007). This provides an argument that Nox mediated inactivation of SAM synthetase is required to keep GSH at lower levels, since cellular redox state is shifted towards higher oxidation levels during infection.

Other enzymes involved in amino acid metabolism are FG00049.1 probable branched chain amino acid transferase (BCAT) and FG10118.1 probable ketol-acid reductoisomerase (KARI). BCATs catalyze transamination of leucine, isoleucine, and valine to yield acetyl CoA, NADH and $FADH_2$ via glucose and/or ketone bodies. FG00049.1 was identified to contain one reactive cysteine. Coles et al (2009) reported that BCATs undergo S-Nitrosoglutathione inactivation. The presence of a redox-active dithiol/disulfide center has been suggested in the regulation of the human mitochondrial BCAT isozyme, where one thiol group acts as the "resolving cysteine", permitting formation of a mixed disulfide bond (Conway et al., 2004). KARI requires Mg^{2+} and

NADPH for activity (Ghaemmaghami et al., 2003). There is one reversibly oxidized cysteine detected in this enzyme.

Two reversibly modified cysteines were detected in FG09321.1 Acetoacetyl-CoA thiolase. This enzyme is known to be susceptible to iodoacetamide inhibition. Redox regulation of plant peroxisomal thiolase structure and activity, and loss of mitochondrial thiolase activity under oxidative stress have been recognized (Pye et al., 2010; Santhanam et al., 2007). Thiolase have a dual function in catabolism of acetoacetyl-CoA and regulating the production of ketone bodies. Secondly it catalyzes breakdown of the isoleucine. End products are acetyl CoA, NADH and $FADH_2$. The redox effect on these enzymes is not documented. It is postulated that reversible inactivation of BCATs and thiolase might be a cellular mechanism to reduce flux towards TCA cycle while KARI inhibition is to increase NADPH availability to Nox enzymes by reducing the activity of enzymes utilizing NADPH.

FG10737.1 probable Adenylate kinase (ADK) ws identified with one reversibly modified thiol residue Cys^{116}. ADK and downstream AMP signalling monitors the cellular energy state and responds to metabolic stress (Dzeja and Terzic 2009; Juhnke et al., 2000). ADK forms a conformation with DTT resulting in gradual decline in its activity *in vitro* (Xia Li et al., 2001). Cysteine may not participate directly in substrate binding but has putative role in stabilizing secondary and tertiary structures of native AK.

5.2.2 Cellular anti-oxidant system

Thiol-oxidoreductases are the anti-oxidant enzymes that are characterized by the presence of CxxC, CxxS or SxxC motifs and utilize cysteine groups for defense against elevated ROS. In our study we identified three thiol-oxidoreductases FG00353.1

peroxiredoxin 5 (Prx5), FG01246.1 protein disulfide isomerase (PDI) and FG08677.1 alkyl hydroperoxide reductase 1 (Ahp1) undergoing reversible oxidation. The cysteine motifs SxxC, CxxC and CxxS were detected in significant peptides K.TSNGYIVGVPAAF**SGTC**SSK.H of FG00353.1, K.SNDLVLAEFFAPW**CGHC**K.A of FG01246.1 and K.VVLISVPGAFTPT**CSAS**HVPSYVENIEQIK.A of FG08677.1 respectively. Prx5 are oxidised at their active cysteine by a peroxide substrate; to reduce H_2O_2, alkyl hyperoxides and peroxynitrite in an NADPH-, Trx/TrxR-dependent manner. Active cysteine forms an intra/intermolecular reversible disulfide bond with another cysteine (resolving cysteine) with sulfenic acid as an intermediate form (Thon et al., 2007; Wood et al., 2003). Eukaryotic Prxs not only act as antioxidants, but also appear to regulate hydrogen peroxide-mediated signal transduction by keeping resting levels of hydrogen peroxide low, while permitting higher levels during signal transduction. Ahp1 also belongs to the peroxiredoxin family. In yeast, Ahp1p was associated with oxidative stress protection, to protect cells against metal ion toxicity and glutathione diminution (Nguyên-nhu and Knoops 2002). PDI catalyzes the formation and isomerization of disulfides during oxidative protein folding. The major route for oxidizing thiols to disulfides is through cyclic reduction and oxidation of PDI at its active site cysteines. It is thought that these enzymes are oxidized by the flavoprotein ER oxidoreductin 1 (Ero1), which couples disulphide formation with reduction of oxygen to form hydrogen peroxide (Schwaller et al., 2003). Studies with Ero1-deficient mammalian cells exhibited Ero1-independent pathways to disulfide bond formation, with Peroxiredoxin IV (PrxIV) as a candidate oxidant of PDI in the presence of H_2O_2 (Zito et al., 2010). Several members of the PDI family are able to directly reduce PrxIV disulphides and in the process become

oxidized (Tavender et al., 2010). The oxidation of PDI by peroxiredoxin is a highly efficient process and demonstrates that oxidation by Nox generated H_2O_2 can be coupled to disulphide formation.

5.2.3 ROS Scavengers and involvement in virulence

Two enzymes reported as virulence factors FG04558.1 carbonic anhydrase (CA) and FG04826.1 protein similar to mannitol dehydrogenase (MDH) were identified as Nox targets containing one redox responsive cysteine. CA catalyzes the interconversion of carbon dioxide and bicarbonate, which is indispensable for multiple biological processes like photosynthesis, respiration, CO_2 transport and pH homeostasis. Several reports demonstrated that CA governed CO_2-sensing system is involved in the regulation of sexual development in fungal pathogens (Elleuche and Pöggeler 2009; Bahn et al., 2005). Association between cAMP signaling and CO_2/HCO_3^- balance exposes CO_2 sensing to be an intermediary in fungal pathogenesis (Klengel et al., 2005). CA was found to be redox regulated through H_2O_2 and peroxyl radicals via oxidized protein sulfhydryl residues (Mallis et al., 2002). Studies suggested that CA functions as a ROS scavenger and participates in protecting cells from oxidative damage (Lee et al., 2004b). MDH is involved in catabolism of mannitol. Mannitol is a major stress molecule of fungi, abundant in mycelia during infection (Ruijter et al., 2003). Mannitol production by fungal phytopathogens is an important virulence factor and secretion of mannitol occurs during disease establishment to quench ROS mediated plant defenses (Aguirre et al., 2006). Reversible switching of the activity of MDH by Nox generated ROS could be a temporal mechanism to regulate mannitol secretion during the infection process.

5.2.4 Regulatory molecules:

14-3-3 proteins are components of signalling pathways conserved in all eukaryotes. They are able to bind a host of functionally varied signalling proteins, such as kinases, phosphatases, and transmembrane receptors. In fungi, 14-3-3 proteins are involved in cell cycle regulation, cytokinesis, filamentous growth and virulence (Hurtado and Rachubinski 2002). In *Candida albicans*, a mutation in the 14-3-3 protein (Bmh1p) affected a variety of different pathways associated with virulence, including formation of filaments and host interaction (Kelly et al., 2009). FG06847.1 a homolog of 14-3-3 protein was identified as a redox target in our study. Activation of two 14-3-3 proteins, general regulatory factor 10 (GRF10 or GFε) and GF14λ involved in brassinosteroid signaling, by buthionine sulfoximine (BSO) and H_2O_2 treatments has been reported (DeLille et al., 2001). Brassinosteroids are plant hormones associated with cellular growth and abiotic and biotic stresses tolerance. Application of BSO depletes glutathione and enables a build-up of endogenous H_2O_2 similar to the result of Nox activity in endogenous ROS production. Although phosphorylation was initially believed to be regulating 14-3-3 binding, some isoforms do not require phosphorylation (van Hemert et al., 2001). It is suggested that redox modification of 14-3-3 proteins could alter protein conformation, thus modulating protein–protein interaction and activating/deactivating signalling pathways. Kim et al (2009) demonstrated that the ROS generated by NADPH oxidases engages in regulation of cytoskeletal organization and cell migration via oxidation of a 14-3-3 protein.

5.2.5 Proteins with diverse roles

FG08077.1 Probable NADPH dehydrogenase (NDH) is found to be involved in Nox mediated redox response. NDH is located in the outer face of the inner mitochondrial membrane and transfers an electron from cytosolic NADPH to quinones. It realizes the turnover of NADPH into an NADP(+) pool in a calcium-dependent manner (Melo et al., 2004). Redox inactivation of NDH would be a mechanism to increase NADPH flux towards ROS generating Nox activity. FG11142.1 Dihydrodiol dehydrogenase is involved in the metabolism of polycyclic aromatic hydrocarbons. It is reported to be extremely sensitive to SH reagents such as NEM and nitrobenzoic acid, which caused time- and concentration-dependent reversible enzyme inactivation (Nanjo et al., 1995). This suggests that alteration of FG11142.1 activity depends on the modulation through a thiol/disulphide exchange reaction. FG02712.1 Cytochrome c oxidase is the terminal enzyme of the mitochondrial electron transport chain. The redox effect on this enzyme is not known and there is no evidence of links with virulence related functions. It is postulated that redox regulation of FG02712.1, which is capable of using H_2O_2 as a co-substrate, might result in an increase of hydrogen peroxide availability in the fungal cultures. FG03535.1 *Fusarium* Tri4 (Trichodiene oxygenase) belongs to cytochrome P450 monooxygenase family and catalyzes four successive oxygenation reactions in trichothecene biosynthesis (McCormick et al., 2006). Cytochrome P450 enzymes are some of the most versatile redox proteins known. Self-inactivation of these enzymes during catalytic turnover accompanies oxidation of SH-groups by H_2O_2 (Karuzina et al., 1999).

FG01425.1 is a subunit of H+ ATPase which was subjected to reversible redox regulation in our study. Fungal plasma membrane H^+-ATPase is a proton pump which participates in the physiology of intracellular pH regulation and nutrient uptake by facilitating ATP-dependent H+ removal. Alteration of intracellular pH occurs during fungal pathogenesis and is believed to regulate secretion of toxins; DON synthesis largely depends on low extracellular pH as is evident by acidification of medium *in vitro*, although it is unknown if an acidic pH is essential *in planta* for initiation of toxin production (Gardiner et al., 2009). High pH also stabilizes superoxide, as required to maintain ROS induced signalling.

Protein ecm33 (FG10089.1) is likely a protein functioning in sporulation and conidial cell wall biosynthesis. There is no report on redox regulation of FG10089.1 so far. It is postulated that activity of this enzyme is switched on as the fungal cell undergoes differentiation and conidial germination starts in consequence of redox signals. A translation elongation factor EF-2 (FG09574.1) and a heat shock protein Hsp70 (FG00838.1) were identified to undergo reversible thiol modification. In *E. coli* Hsp33 was reported to alter conformation upon oxidation to a state that bound protein substrates and prevented protein aggregation (Graumann et al., 2001). Several proteins involved in ribosome biogenesis have been reported as signalling components, undergoing redox modification and phosphorylation-dephosphorylation (Rampitsch et al., 2010; Lee et al., 2004; Mirzaei et al., 2008; Fratelli et al., 2002). We identified multiple ribosome subunits such as FG07292.1, FG00802.1, Fg06019.1, Fg07186.1, FG10246.1 and FG10905.1 to be reversibly modified at cysteine residues.

5.2.6 Tentative scheme of cellular events affected by Nox activity

In view of the findings obtained, Figure 5.1 depicts the tentative scheme of cellular events affected by Nox activity in *F. graminearum*. As a consequence of nutritional stress, Nox enzymes were activated to produce ROS by using cellular NADPH. The balance between cellular ROS and anti-oxidants was altered in favour of higher oxidizing levels, resulting in reversible thiol-oxidation of metabolic enzymes, antioxidant enzymes and regulatory proteins. Proteomics findings suggested that glycolysis and TCA cycle were inhibited and metabolic flux was directed towards pentose phosphate pathway to increase cellular NADPH levels. Cellular antioxidant system was depleted to maintain higher levels of ROS required for developmental events such as production of fruiting bodies and ascospore germination subsequently leading to disease progression. Proteins involved in nitrogen assimilation; metabolism of amino-acids and nucleic acids; ribosome biogenesis and protein synthesis were also found to be redox modified.

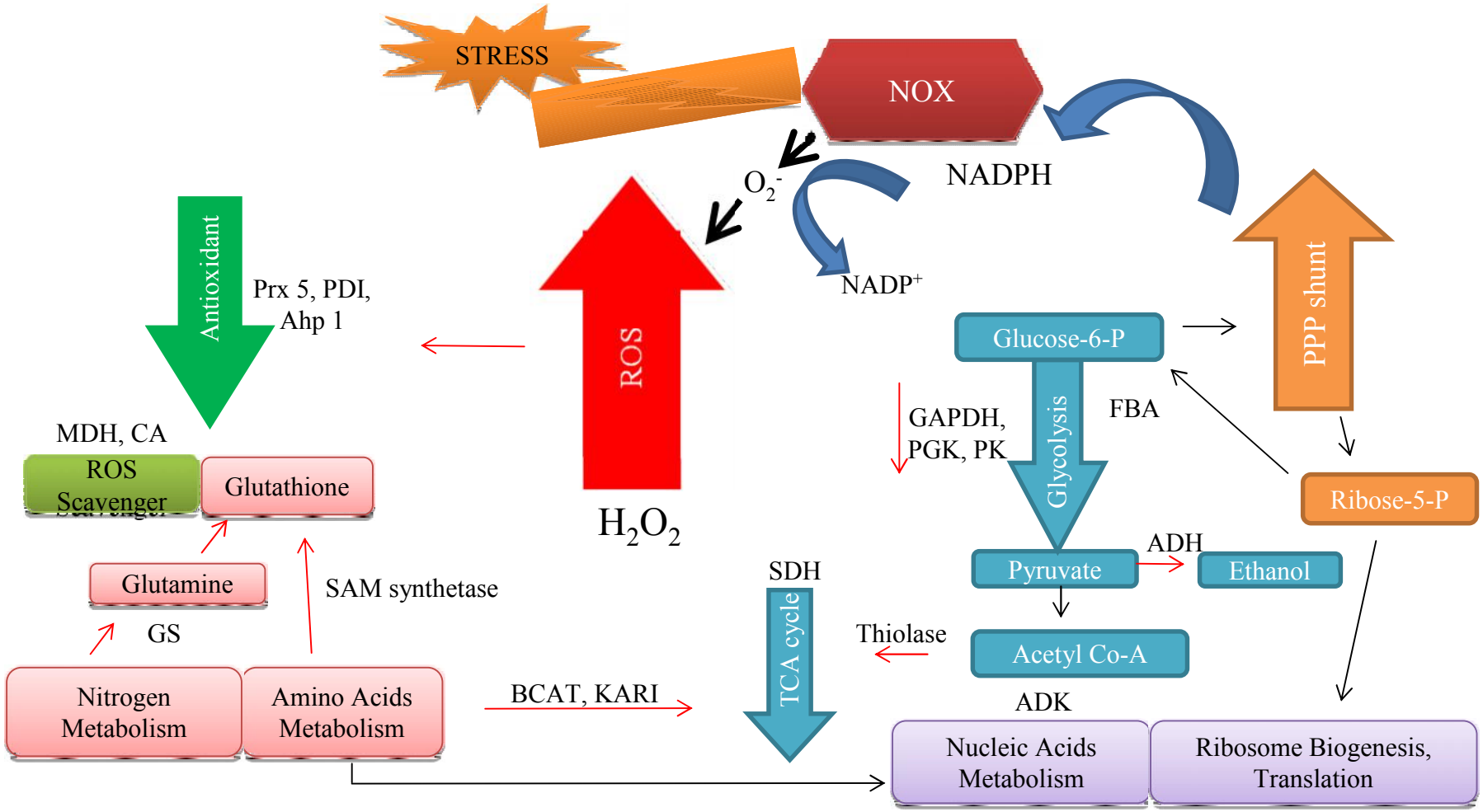


Figure 5.1 Tentative scheme of cellular events affected by Nox regulated increase in ROS levels in *F. graminearum*. Nox enzymes, activated by nutritional stress, used cellular NADPH to produce ROS. Glycolysis and TCA cycle were inhibited (Blue thick downwards arrow) and metabolic flux was directed towards Pentose phosphate pathway (PPP) to increase cellular NADPH levels (Orange thick upwards arrow). Cellular antioxidant system was depleted to maintain higher levels of ROS required for disease progression (Green thick downwards arrow). Connecting thin arrow in red indicates inhibition of a pathway due to reversible thiol modification of the enzyme involved (labeled). Nitrogen assimilation; amino-acids and nucleic acids metabolism; ribosome biogenesis and protein synthesis were also found to be affected in the study (shown in boxes). Details are discussed in the text.

CONCLUSION

Chapter 6: Conclusion

Steady states in a cell are maintained by equilibrium between ROS generation (by Nox enzymes and mitochondria) and removal (by antioxidant enzymes). Changes in ROS equilibrium act as a signal for multicellular development, spore germination and virulence in filamentous fungi. Nox enzymes provide the regulated mechanism to employ ROS for achieving the necessary changes in fungal metabolic and regulatory pathways. In this study, the first redox-disulfide proteomic map of *F. graminearum* was established. We demonstrated *in vitro* that during conditions of FHB induction increase in ROS modified redox status and affected the cellular events. Proteomic data obtained indicated that total of 35 proteins (at structure and/or activity levels) were altered through reversible oxidative modifications at their cysteine residues. Approximately 34% of the identified proteins were involved in fungal metabolic pathways, prominently in carbon and nitrogen assimilation. Evidence was found that glycolytic enzymes were reversibly oxidised at their thiol residues to slow down glycolysis during disease induction. The study also suggested links between redox signalling and the components involved in perception of nutrient information for the pathogen. 20% of the candidate proteins participated in regulation of redox potential through electron transport. 9% of the identified proteins belong to thiol-oxidoreductases which eliminate ROS from the cell. Reversible inactivation of anti-oxidants may be needed to maintain ROS-signalled induction of virulence. Proteins with potential involvement in pathogenesis related functions, demonstrated in *F. graminearum* or other filamentous fungi, including Tri4 (Trichodiene oxygenase) involved in tricothecene biosynthesis; Protein ecm33 functioning in sporulation and conidial cell wall biosynthesis; H+ ATPase, Carbonic

anhydrase and Manntiol dehydrogenase were found to undergo thiol modification. In view of this, Nox enzymes appear to play a significant role during FHB induction, through their action as a redox switch for modifying a set of proteins involved in the corresponding physiological processes. Taken together, the findings support that redox mediated changes in fungal metabolism and developmental processes are important in triggering *F. graminearum* pathogenesis.

REFERENCES

REFERENCES

Abramson, D., Clear, R. M. and Nowicki, T. W. 1987. *Fusarium* species and trichothecene mycotoxins in suspect samples of 1985 Manitoba wheat. Canadian Journal of Plant Science. 67: 611-619.

Aguirre, J., Ríos-Momberg, M., Hewitt, D. and Hansberg, W. 2005. Reactive oxygen species and development in microbial eukaryotes. Trends in Microbiology. 13(3): 111-118.

Aguirre, J., Hansberg, W. and Navarro, R. 2006. Fungal responses to reactive oxygen species. Medical Mycology. 44: S101-S107.

Alvarez, S., Wilson, G. H. and Chen, S. Determination of in vivo disulfide-bonded proteins in *Arabidopsis*. Journal of chromatography. B, Analytical technologies in the biomedical and life sciences. 877(1-2): 101-104.

Anderson, L. and Hunter, C. L. 2006. Quantitative mass spectrometric multiple reaction monitoring assays for major plasma proteins. Molecular and Cellular Proteomics. 5(4): 573-88.

Bahn, Y. S., Cox, G. M., Perfect, J. R. and Heitman, J. 2005. Carbonic anhydrase and CO_2 sensing during *Cryptococcus neoformans* growth, differentiation, and virulence. Current Biology. 15(22): 2013-2020.

Bai, G. H., Desjardins, A. E., and Plattner, R. D. 2002. Deoxynivalenol nonproducing *Fusarium graminearum* causes initial infection, but does not cause disease spread in wheat spikes. Mycopathologia. 153: 91-98.

Bai, G.H., and Shaner, G. 2004. Management and resistance in wheat and barley to Fusarium head blight. Annual review of Phytopathology. 42: 135-161.

Barelle, C. J., Priest, C. L., Maccallum, D. M., Gow, N. A., Odds, F. C. and Brown, A. J. 2006. Niche-specific regulation of central metabolic pathways in a fungal pathogen. Cellular Microbiology. 8(6): 961-971.

Bennett, J. W. and Klich, M. 2003. Mycotoxins. Clinical Microbiology Reviews. 16(3): 497-516.

Beyer, M. and Verreet, J.-A. 2005. Germination of *Gibberella zeae* ascospores as affected by age of spores after discharge and environmental factors. European Journal of Plant Pathology. 111: 381-389.

Boutigny, A. L., Richard, F. F. and Barreau, C. 2008. Natural mechanisms for cereal resistance to the accumulation of *Fusarium* trichothecenes. European Journal of Plant Pathology. 121: 411-423.

Bowyer, P., Mueller, E. and Lucas, J. 2000. Use of an isocitrate lyase promoter-GFP fusion to monitor carbon metabolism of the plant pathogen *Tapesia yallundae* during infection of wheat. Molecular Plant Pathology. 1: 253 – 262.

Buchanan, B. B. and Balmer, Y. 2005. Redox regulation: a broadening horizon. Annual Review of Plant Biology. 56: 187-220.

Bushnell, W. R., Hazen, B. H., and Pritsch, C. 2003. Histology and Physiology of Fusarium Head Blight. *In* Fusarium Head Blight of Wheat and Barley, Leonard, K.J., Bushnell, W.R., Eds., The American Phytopathological Society: St. Paul, Minnesota, USA, 2003, Chapter 3, pp.44-83.

Bykova, N. V., Hoehn, B., Rampitsch, C., Banks, T., Stebbing, J. A., Fan, T. and Knox, R. 2011. Redox-sensitive proteome and antioxidant strategies in wheat seed dormancy control. Proteomics. 11(5): 865-882.

Calabrese, M. G., Mamone, G , Cairab, S., Ferrantia, P. and Addeoa, F. 2009. Quantitation of lysinoalanine in dairy products by liquid chromatography-mass spectrometry with selective ion monitoring. Food Chemistry. 116(3): 799-805.

Cano-Domínguez, N., Alvarez-Delfín, K., Hansberg, W. and Aguirre, J. 2008. NADPH oxidases NOX-1 and NOX-2 require the regulatory subunit NOR-1 to control cell differentiation and growth in *Neurospora crassa*. Eukaryotic Cell. 7(8):1352-1361.

Carter, J. P., Rezanoor, H. N, Holden, D., Desjardins, A. E, Plattner, R. D and Nicholson P. 2002. Variation in pathogenicity associated with the genetic diversity of *Fusarium graminearum*. European Journal of Plant Pathology. 108: 573-583.

Chen, E. I. and Yates, J. R. 2007. Cancer proteomics by quantitative shotgun proteomics. Molecular Oncology. 1(2): 144–159.

Coles, S. J., Easton, P., Sharrod, H., Hutson, S. M., Hancock, J., Patel, V. B. and Conway, M. E. 2009. S-Nitrosoglutathione inactivation of the mitochondrial and cytosolic BCAT proteins: S-nitrosation and S-thiolation. Biochemistry. 48(3): 645-656.

Conway, M. E., Poole, L. B. and Hutson, S. M. 2004. Roles for cysteine residues in the regulatory CXXC motif of human mitochondrial branched chain aminotransferase enzyme. Biochemistry. 43(23): 7356-7364.

Couture, L. 1982. Receptivity of spring wheat cereal cultivars to contamination of grain in the in florescence by *Fusarium* spp. Canadian Journal of Plant Science. 62: 29-34.

Crespo, J.L., Powers, T., Fowler, B., and Hall, M.N. 2002. The TOR-controlled transcription activators GLN3, RTG1, and RTG3 are regulated in response to intracellular levels of glutamine. Proceedings of the National Academy of Sciences (USA). 99: 6784–6789.

Cumagun, C. J. R., Bowden, R. L., Jurgenson, J. E., Leslie, J. F., and Miedaner, T. 2004. Genetic mapping of pathogenicity and aggressiveness of *Gibberella zeae* (*Fusarium graminearum*) toward wheat. Phytopathology. 94: 520-526.

Cuomo, C. A., Güldener, U., Xu, J.-R., Trail, F., Turgeon, B. G. et al. 2007. The Fusarium graminearum genome reveals a link between localized polymorphism and pathogen specialization. Science. 317(5843): 1400–1402.

De Jong, J. C., McCormack, B. J., Smirnoff, N. and Talbot, N. J. 1997. Glycerol generates turgor in rice blast. Nature. 389: 244-245.

De Lille, J. M., Sehnke, P. C. and Ferl, R. J. 2001. The Arabidopsis 14-3-3 family of signaling regulators. Plant Physiology. 126: 35–38.

Desjardins, A. E. 2006. Fusarium mycotoxins-chemistry, genetics, and biology. The American Phytopathological Society Press.

Desjardins, A. E., Proctor, R. H., Bai, G. H., McCormick, S. P., Shaner, G., Buechley, G., and Hohn, T. M. 1996. Reduced virulence of trichothecene-nonproducing mutants of *Gibberella zeae* in wheat field tests. Molecular Plant–Microbe Interactions. 9: 775-781.

Di Pietro, A., García-MacEira, F. I., Méglecz, E., Roncero, M. I. 2001. A MAP kinase of the vascular wilt fungus *Fusarium oxysporum* is essential for root penetration and pathogenesis. Molecular Microbiology. 39(5):1140-1152.

Diebold, B. A. and Bokoch, G. M. 2001. Molecular basis for Rac2 regulation of phagocyte NADPH oxidase. Nature Immunology. 2: 211–215.

Ding, L., Li, L., Tao, P., Yang, J. and Zhang, Z. 2002. Quantitation of tamsulosin in human plasma by liquid chromatography–electrospray ionization mass spectrometry. Journal of Chromatography B: Analytical Technology in Biomedicine and Life Sciences. 767: 75–81.

Dzeja, P. and Terzic, A. 2009. Adenylate kinase and AMP signaling networks: Metabolic monitoring, signal communication and body energy sensing. International Journal of Molecular Sciences. 10(4): 1729-1772.

Egan M. J., Wang Z.Y., Jones M. A., Smirnoff N. and Talbot N. J. 2007. Generation of reactive oxygen species by fungal NADPH oxidases is required for rice blast disease. Proceedings of the National Academy of Sciences (USA). 104(28): 11772-11777.

Elleuche, S. and Pöggeler, S. 2009. Beta-carbonic anhydrases play a role in fruiting body development and ascospore germination in the filamentous fungus *Sordaria macrospora*. PLoS One. 4(4):e5177.

Fang, R., Elias, D. A., Monroe, M. E., Shen, Y., McIntosh, M., Wang, P., Goddard, C. D., Callister, S. J., Moore, R. J., Gorby, Y. A., Adkins, J. N., Fredrickson, J. K., Lipton, M. S. and Smith, R. D. 2006. Differential label-free quantitative proteomic analysis of *Shewanella oneidensis* cultured under aerobic and suboxic conditions by accurate mass and time tag approach. Molecular and Cellular Proteomics. 5(4): 714-725.

Fenn, J. B., Mann, M., Meng, C. K., Wong, S. F. and Whitehouse, C. M. 1989. Electrospray ionization for mass spectrometry of large biomolecules. Science. 246(4926): 64–71.

Foreman, J., Demidchik, V., Bothwell, J. H., Mylona, P., Miedema, H., Torres, M. A., Linstead, P., Costa, S., Brownlee, C., Jones, J. D. G., Davies, J. M. and Dolan, L. 2003. Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. Nature. 422: 442–446.

Fratelli, M., Demol, H., Puype, M., Casagrande, S., Eberini, I. and Salmona, M. 2002. Identification by redox proteomics of glutathionylated proteins in oxidatively stressed human T lymphocytes. Proceedings of the National Academy of Sciences (USA). 99: 3505-3510.

Gardiner, D. M., Osborne, S., Kazan, K. and Manners, J. M. 2009. Low pH regulates the production of deoxynivalenol by *Fusarium graminearum*. Microbiology. 155(9): 3149-3156.

Ghaemmaghami, S., Huh, W. K., Bower, K., Howson, R. W., Belle, A., Dephoure, N., O'Shea, E. K. and Weissman, J. S. 2003. Global analysis of protein expression in yeast. Nature. 425(6959): 737-741.

Giesbert, S., Schürg, T., Scheele, S. and Tudzynski, P. 2008. The NADPH oxidase Cpnox1 is required for full pathogenicity of the ergot fungus *Claviceps purpurea*. Molecular Plant Pathology. 9(3): 317-327.

Gilbert, J., Woods, S. M., Kromer, U. 2008. Germination of ascospores of *Gibberella zeae* after exposure to various levels of relative humidity and temperature. Phytopathology. 98(5): 504-508.

Gilbert, J. and Fernando, W.G. D. 2004. Epidemiology and biological control of *Gibberella zeae/Fusarium graminearum.* Canadian Journal Plant Pathology. 26: 464-472.

Gilbert, J. and Tekauz, A. 2000. Review: Recent developments in research on Fusarium head blight of wheat in Canada. Canadian Journal Plant Pathology. 22: 1-8.

Gilbert, J., Tekauz, A., Kaethler, R., Mueller, E. and Kromer, U. 1995. Occurrence of *Fusarium* head blight in Manitoba in 1994. Canadian Plant Disease Survey. 74: 77-78.

Godon, C., Lagniel, G., Lee, J., Buhler, J. M., Kieffer, S., Perrot, M., Boucherie, H., Toledano, M. B. and Labarre, J. 1998. The H_2O_2 stimulon in *Saccharomyces cerevisiae*. The Journal of Biological Chemistry. 273: 22480-22489.

González-Andrade, M., Rivera-Chávez, J., Sosa-Peinado, A., Figueroa, M., Rodríguez-Sotres, R. and Mata, R. 2011. Development of the fluorescent biosensor hCalmodulin (hCaM)L39C-monobromobimane(mBBr)/V91C-mBBr, a novel tool for discovering new calmodulin inhibitors and detecting calcium. Journal of Medicinal Chemistry. 54(11): 3875-3884.

Görg. A., Weiss, W. and Dunn, M. J. 2004. Current two-dimensional electrophoresis technology for proteomics. Proteomics. 4(12): 3665-3685.

Graumann, J., Lilie, H., Tang, X., Tucker, K. A., Hoffmann, J. H., Vijayalakshmi, J., Saper, M., Bardwell, J. C. and Jakob, U. 2001. Activation of the redox-regulated molecular chaperone Hsp33--a two-step mechanism. Structure. 9: 377–387.

Guenther, J. C. and Trail, F. 2005. The development and differentiation of *Gibberella zeae* (anamorph: *Fusarium graminearum*) during colonization of wheat. Mycologia. 97: 229-237.

Güldener U., Mannhaupt G., Münsterkötter M., Haase D., Oesterheld M., Stümpflen V., Mewes H. W. and Adam G. 2006a. FGDB: a comprehensive fungal genome resource on the plant pathogen *Fusarium graminearum*. Nucleic Acids Research. 34: D456–D458.

Güldener U., Seong K. Y., Boddu J., Cho S., Trail F., Xu J. R., Adam G., Mewes H. W., Muehlbauer G. J. and Kistler H. C. 2006b. Development of a *Fusarium graminearum* Affymetrix GeneChip for profiling fungal gene expression in vitro and in planta. Fungal Genetics and Biology. 43(5): 316-25.

Gygi, S. P., Rist, B., Gerber, S. A., Turecek, F., Gelb, M. H. and Aebersold, R. 1999. Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. Nature Biotechnology. 17: 994–999.

Hansberg, W. and Aguirre, J. 1990. Hyperoxidant states cause microbial cell differentiation by cell isolation from dioxygen. Journal of Theoretical Biology.142(2): 201-221.

Harris, L. J., Alexander, N. J., Saparno, A., Blackwell, B., McCormick, S. P., Desjardins, A. E., Robert, L. S., Tinker, N., Hattori, J., Piché, C., Schernthaner, J. P., Watson, R. and Ouellet, T. 2007. A novel gene cluster in *Fusarium graminearum* contains a gene that contributes to butenolide synthesis. Fungal Genetics and Biology. 44: 293–306.

Hederstedt, L. and Hedén, L. O. 1989. New properties of *Bacillus subtilis* succinate dehydrogenase altered at the active site. The apparent active site thiol of succinate oxidoreductases is dispensable for succinate oxidation. Biochemical Journal. 260(2): 491-497.

Hennebert, G. L. 1987. Pleoanamorphy and its nomenclatural problem. In: Pleomorphic Fungi: The Diversity and its Taxonomic Implications (ed. J. Sugiyama). Kodansha Ltd., Tokyo: 263-290.

Hohn, T. M and Beremand, P. D. 1989. Isolation and nucleotide sequence of a sesquiterpene cyclase gene from the trichothecene-producing fungus *Fusarium sporotrichiodies*. Gene. 79: 131-138.

Hou, Z., Xue, C., Peng, Y., Katan, T. Kistler H. C. and Xu J. R. 2002. A mitogen-activated protein kinase gene (MGV1) in *Fusarium graminearum* is required for female fertility, heterokaryon formation, and plant infection. Molecular Plant–Microbe Interactions. 15: 1119–1127.

Hoyal, C. R., Gutierrez, A., Young, B. M., Catz, S. D., Lin, J. H., Tsichlis P. N. and Babior, B.M. 2003. Modulation of $p47^{phox}$ activity by site-specific phosphorylation: Akt-dependent activation of the NADPH oxidase. Proceedings of the National Academy of Sciences (USA). 100: 5130–5135.

Hurtado, C. A. and Rachubinski, R. A. 2002. YlBMH1 encodes a 14-3-3 protein that promotes filamentous growth in the dimorphic yeast *Yarrowia lipolytica*. Microbiology. 148(11): 3725-3735.

Jaffrey, S. R. and Snyder, S. H. 2001. The biotin switch method for the detection of S-nitrosylated proteins. Science Signaling. 2001(86): l1.

Jakob, U., Muse, W., Eser, M. and Bardwell, J. C. 1999. Chaperone activity with a redox switch. Cell 96: 341–352.

Jansen, C., von Wettstein, D., Schäfer, W., Kogel, K. H., Felk, A., Maier, F. J. 2005. Infection patterns in barley and wheat spikes inoculated with wild-type and trichodiene synthase gene disrupted *Fusarium graminearum*. Proceedings of the National Academy of Sciences (USA). 102(46): 16892-16897.

Jenczmionka, N. J., Maier, F. J., Losch, A. P. and Schafer, W. 2003. Mating, conidiation and pathogenicity of *Fusarium graminearum*, the main casual agent of the head-blight disease of wheat, are regulated by the MAP kinase *gpmk1*. Current Genetics. 43: 87–95.

Jiang, F., Zhang, Y., Dusting, G. J. 2011. NADPH oxidase-mediated redox signaling: Roles in cellular stress response, stress tolerance, and tissue repair. Pharmacological Reviews 63(1): 218-242.

Jorge, I., Casas, E. M., Villar, M., Ortega-Pérez, I., López-Ferrer, D., Martínez-Ruiz, A., Carrera, M., Marina, A., Martínez, P., Serrano, H., Cañas, B., Were, F., Gallardo, J. M., Lamas, S., Redondo, J. M., García-Dorado, D. and Vázquez, J. 2007. High-sensitivity analysis of specific peptides in complex samples by selected MS/MS ion monitoring and linear ion trap mass spectrometry: Application to biological studies. Journal of Mass Spectrometry. 42: 1391–1403.

Juhnke, H., Charizanis, C., Latifi, F., Krems, B. and Entian, K. D. 2000. The essential protein fap7 is involved in the oxidative stress response of *Saccharomyces cerevisiae*. Molecular Microbiology. 35(4): 936-948.

Kang, J. G., Paget, M. S., Seok, Y. J., Hahn, M. Y., Bae, J. B., Hahn, J. S., Kleanthous, C., Buttner, M. J. and Roe, J. H. 1999. RsrA, an anti-sigma factor regulated by redox change. The EMBO Journal. 18(15): 4292-4298.

Kang, Z. and Buchenauer, H. 2000a. Cytology and ultrastructure of the infection of wheat spikes by *Fusarium culmorum*. Mycological Research. 104: 1083-1093.

Kang, Z. and Buchenauer, H. 2000b. Ultrastructural and cytochemical studies on cellulose, xylan, and pectin degradation in wheat spikes infected by *Fusarium culmorum*. Journal of Phytopathology. 148: 263-275.

Karuzina, I. I., Zgoda, V. G., Kuznetsova, G. P., Samenkova, N. F. and Archakov, A. I. 1999. Heme and apoprotein modification of cytochrome P450 2B4 during its oxidative inactivation in monooxygenase reconstituted system. Free Radical Biology and Medicine. 26(5-6): 620-632.

Kawasaki, L. and Aguirre, J. 2001. Multiple catalase genes are differentially regulated in Aspergillus nidulans. J Bacteriol. 183(4): 1434-1440.

Kawasaki, L., Sánchez, O., Shiozaki, K. and Aguirre, J. 2002. SakA MAP kinase is involved in stress signal transduction, sexual development and spore viability in *Aspergillus nidulans*. Molecular Microbiology. 45: 1153–1163.

Kelly, M. N., Johnston, D. A., Peel, B. A., Morgan, T. W., Palmer, G. E. and Sturtevant, J. E. 2009. Bmh1p (14-3-3) mediates pathways associated with virulence in *Candida albicans*. Microbiology. 155(5): 1536-1546.

Khan, M. R. and Doohan, F. M. 2009. Bacterium-mediated control of Fusarium head blight disease of wheat and barley and associated mycotoxin contamination of grain. Biological control. 48: 42-47.

Kim, B. J., Hood, B. L., Aragon, R. A., Hardwick, J. P., Conrads, T. P., Veenstra, T. D. and Song, B. J. 2006. Increased oxidation and degradation of cytosolic proteins in alcohol-exposed mouse liver and hepatoma cells. Proteomics. 6: 1250–1260.

Kim, J. S., Huang, T. Y. and Bokoch, G. M. 2009. Reactive oxygen species regulate a slingshot-cofilin activation pathway. Molecular Biology of the Cell. 20(11): 2650-2660.

Kimura, M., Tokai, T., O'Donnell, K., Ward, T. J., Fujimura, M., Hamamoto, H., Shibata, T., Yamaguchi, I. 2003. The trichothecene biosynthesis gene cluster of *Fusarium graminearum* F15 contains a limited number of essential pathway genes and expressed non-essential genes. FEBS Letters. 539: 105-110.

Kitteringham, N. R., Jenkins, R. E., Lane, C. S, Elliott, V. L., Park, B. K. 2009. Multiple reaction monitoring for quantitative biomarker analysis in proteomics and metabolomics. Journal of chromatography. B, Analytical technologies in the biomedical and life sciences. 877(13):1229-1239.

Klaunig, J. E. and L. M. Kamendulis. 2004. The role of oxidative stress in carcinogenesis. Annual Review of Pharmacology and Toxicology. 44: 239-267.

Klengel, T., Liang, W. J., Chaloupka, J., Ruoff, C., Schröppel, K., Naglik, J. R., Eckert, S. E., Mogensen, E. G., Haynes, K., Tuite, M. F., Levin, L. R., Buck, J. and Mühlschlegel, F. A. 2005. Fungal adenylyl cyclase integrates CO_2 sensing with cAMP signaling and virulence. Current Biology. 15(22): 2021-2026.

Klix, B. M., Verreet, J. A, and Beyer, M. 2007. Comparison of the declining triazole sensitivity of *Gibberella zeae* and increased sensitivity achieved by advances in triazole fungicide development. Crop protection. 26: 683-690.

Kojima, K., Kikuchi, T., Takano, Y., Oshiro, E. and Okuno, T. 2002. The mitogen-activated protein kinase gene MAF1 is essential for the early differentiation phase of appressorium formation in *Colletotrichum lagenarium*. Molecular Plant–Microbe Interactions.15(12):1268-1276.

Komeili, A., Wedaman, K.P., O'Shea, E.K., and Powers, T. 2000. Mechanism of metabolic control: target of rapamycin signaling links nitrogen quality to the activity of the Rtg1 and Rtg3 transcription factors. The Journal of Cell Biology. 151: 863–878.

Lalucque, H. and Silar, P. 2003. NADPH oxidase: an enzyme for multicellularity? Trends in Microbiology. 11: 9–12.

Lamarre, C., LeMay, J. D., Deslauriers, N. and Bourbonnais, Y. *Candida albicans* expresses an unusual cytoplasmic manganese-containing superoxide dismutase (SOD3 gene product) upon the entry and during the stationary phase. The Journal of Biological Chemistry. 276(47): 43784-43791.

Lambeth, J. D. 2004. NOX enzymes and the biology of reactive oxygen. Nature Reviews Immunology. 4(3): 181-189.

Lara-Ortíz, T., Riveros-Rosas, H. and Aguirre, J. 2003. Reactive oxygen species generated by microbial NADPH oxidase NoxA regulate sexual development in *Aspergillus nidulans*. Molecular Microbiology. 50(4): 1241-1255.

Le Moan, N., Clement, G., Le Maout, S., Tacnet, F. and Toledano, M. B. 2006. The *Saccharomyces cerevisiae* proteome of oxidized protein thiols: contrasted functions for the thioredoxin and glutathione pathways. Journal of Biological Chemistry. 281(15): 10420-10430.

Lee, C., Lee, S. M., Mukhopadhyay, P., Kim, S.J., Lee, S.C., Ahn, W. S., Yu, M. H., Storz, G. and Ryu, S. E. 2004a. Redox regulation of OxyR requires specific disulfide bond formation involving a rapid kinetic reaction path. Nature Structural & Molecular Biology. 11(12): 1179-1185.

Lee, K., Lee, J., Kim, Y., Bae, D., Kang, KY., Yoon, S. C. and Lim, D. 2004b. Defining the plant disulfide proteome. Electrophoresis. 25(3): 532-541.

Leichert, L. I. and Jakob, U. 2004. Protein thiol modifications visualized in vivo. PLoS Biology. 2(11): e333.

Lemire, B. D and Oyedotun, K. S. 2002. The *Saccharomyces cerevisiae* mitochondrial succinate:ubiquinone oxidoreductase. Biochimica et Biophysica Acta. 1553(1-2): 102-116.

Lewit-Bentley, A. and Rety, S. 2000. EF-hand calcium-binding proteins, Current Opinion in Structural Biology. 10: 637–643.

Lin, S., Shaler, T. A. and Becker, C. H. 2006. Quantification of intermediate-abundance proteins in serum by multiple reaction monitoring mass spectrometry in a single-quadrupole ion trap. Analytical Chemistry. 78(16): 5762-5767.

Longo, V. D., Gralla, E. B. and Valentine, J. S. 1996. Superoxide dismutase activity is essential for stationary phase survival in *Saccharomyces cerevisiae*. Mitochondrial production of toxic oxygen species in vivo. The Journal of Biological Chemistry. 271(21): 12275-12280.

López-Berges, M. S., Rispail, N., Prados-Rosales, R. C. and Di Pietro, A. 2010. A nitrogen response pathway regulates virulence in plant pathogenic fungi: Role of TOR and the bZIP protein MeaB. Plant Signaling and Behavior. 5(12):1623-1625.

Lowell, B. B. and G. I. Shulman. 2005. Mitochondrial dysfunction and type 2 diabetes. Science. 307(5708): 384-387.

Malagnac, F., Lalucque, H., Lepère, G. and Silar, P. 2004. Two NADPH oxidase isoforms are required for sexual reproduction and ascospore germination in the filamentous fungus *Podospora anserina*. Fungal Genetics and Biology. 41(11): 982-97.

Mallis, R. J., Hamann, M. J., Zhao, W., Zhang, T., Hendrich, S. and Thomas, J. A. 2002. Irreversible thiol oxidation in carbonic anhydrase III: protection by S-glutathiolation and detection in aging rats. Biological Chemistry. 383(3-4): 649-662.

Martínez-Chantar, M. L. and Pajares, M. A. 1996. Role of thioltransferases on the modulation of rat liver S-adenosylmethionine synthetase activity by glutathione. FEBS Letters. 397(2-3): 293-297.

Marzluf, G.A. 1997. Genetic regulation of nitrogen metabolism in fungi. Microbiology and Molecular Biology Reviews. 61**:** 17–32.

Matés, J. M., Segura, J. A., Campos-Sandoval, J. A., Lobo, C., Alonso, L., Alonso, F. J. and Márquez, J. 2009. Glutamine homeostasis and mitochondrial dynamics. The International Journal of Biochemistry & Cell Biology. 41(10): 2051-2061.

Matthies, A. and Buchenauer, H. 2000. Effect of tebuconazole (Folicur) and prochloraz (Sportak) treatments on Fusarium head scab development, yield and deoxynivalenol (DON) content in grains of wheat following artificial inoculation with *Fusarium culmorum.* Journal of Plant Disease and Protection. 107: 33-52.

Mayr, B. M., Kohlbacher, O., Reinert, K., Sturm, M., Gröpl, C., Lange, E., Klein, C. and Huber, C. G. 2006. Absolute myoglobin quantitation in serum by combining two-dimensional liquid chromatography-electrospray ionization mass spectrometry and novel data analysis algorithms. Journal of Proteome Research. 5(2): 414-421.

Mayya, V., Rezual, K., Wu, L., Fong, M. B. and Han, D. K. 2006. Absolute quantitation of multisite phosphorylation by selective reaction monitoring mass spectrometry. Molecular and Cellular Proteomics. 5: 1146-1157.

McCormick, S. P., Harris, L. J., Alexander, N. J., Ouellet, T., Saparno, A., Allard, S. and Desjardins, A. E. 2004. *Tri1* in *Fusarium graminearum* encodes a P450 oxygenase. Applied and Environmental Microbiology. 70(4): 2044-51.

McCormick, S. P., Alexander, N. J. and Proctor, R. H. 2006. *Fusarium* Tri4 encodes a multifunctional oxygenase required for trichothecene biosynthesis. Canadian Journal of Microbiology. 52(7): 636-642.

McDonagh, B., Ogueta, S., Lasarte, G., Padilla, C. A. and Bárcena, J. A. 2009. Shotgun redox proteomics identifies specifically modified cysteines in key metabolic enzymes under oxidative stress in *Saccharomyces cerevisiae*. Journal of Proteomics. 72(4): 677-689.

McMullen, M., Jones, R., and Gallenberg, D. 1997. Scab of wheat and barley: A remerging disease of devastating impact. Plant disease. 81: 1340-1348.

Melo, A. M., Bandeiras, T. M. and Teixeira, M. 2004. New insights into type II NAD(P)H:quinone oxidoreductases. Microbiology and Molecular Biology Reviews. 68(4): 603-616.

Men, L. and Wang, Y. 2007. The oxidation of yeast alcohol dehydrogenase-1 by hydrogen peroxide in vitro. Journal of Proteome Research. 6(1): 216-225.

Mesterhazy Á. 1995. Types and components of resistance to Fusarium head blight of wheat. Plant Breeding. 114: 377-386.

Miller, J. D., Young, J. C., and Sampson D. R. 1985. Deoxynivalenol and Fusarium head blight resistance in spring cereals. Journal of Phytopathology. 113: 359-367.

Miller, J. D. and Blackwell, B. A. 1986. Biosynthesis of 3-acetyldeoxynivalenol and other metabolites by *Fusarium culmorum* HLK 1503 in a stirred jar fermentor. Canadian Journal of Botany. 64: 1–5.

Mirocha, C. J., Pathre, S. V. and Christensen, C. M. 1977. *In*: Zearalenone. Rodricks, J. V., Hesseltine, C. W. and Mehlman, M. A. Eds. Mycotoxins in human and animal health. Pathotox Publishers, Park Forest South, IL, U.S.A.

Mirzaei, H., Baena, B., Barbas, C. and Regnier, F. 2008. Identification of oxidized proteins in rat plasma using avidin chromatography and tandem mass spectrometry. Proteomics. 8(7): 1516-1527.

Moon, K. H., Hood, B. L., Kim, B. J., Hardwick, J. P., Conrads, T. P., Veenstra, T. D. and Song, B. J. 2006. Inactivation of oxidized and S-nitrosylated mitochondrial proteins in alcoholic fatty liver of rats. Hepatology. 44: 1218–1230.

Nakashima, A., Maruki, Y., Imamura, Y., Kondo, C., Kawamata, T., Kawanishi, I., Takata, H., Matsuura, A., Lee, K. S., Kikkawa, U., Ohsumi, Y., Yonezawa, K. and Kamada, Y. 2008. The yeast Tor signaling pathway is involved in G2/M transition via polo-kinase. PLoS One. 3(5):e2223.

Nanjo, H., Adachi, H., Aketa, M., Mizoguchi, T., Nishihara, T. and Terada, T. 1995. The role of cysteine in the alteration of bovine liver dihydrodiol dehydrogenase 3 activity. Biochemistry Journal. 310(1): 101-107.

Nathues, E., Joshi, S., Tenberge, K. B., von den Driesch, M., Oeser, B., Bäumer, N., Mihlan, M. and Tudzynski, P. 2004. CPTF1, a CREB-like transcription factor, is involved in the oxidative stress response in the phytopathogen *Claviceps purpurea* and modulates ROS level in its host *Secale cereale.* Molecular Plant–Microbe Interactions. 17(4): 383-393.

Nganje, W.E., Bangsund, D.A., Leistritz, F.L., Wilson, W.W. and Tiapo, N.M. 2002. Estimating the economic impact of a crop disease: the case of Fusarium head blight in U.S. wheat and barley. 2002 National Fusarium Head Blight Forum Proceedings. East Lansing: Michigan State University, pp. 275–281.

Nguyên-nhu, N. T. and Knoops, B. 2002. Alkyl hydroperoxide reductase 1 protects *Saccharomyces cerevisiae* against metal ion toxicity and glutathione depletion. Toxicology Letters. 135(3): 219-228.

O'Donnell, K., Ward, T. J., Geiser, D. M., Kistler, H. C., and Aoki, T. 2004. Genealogical concordance between the mating type locus and seven other nuclear genes supports formal recognition of nine phylogenetically distinct species within the *Fusarium graminearum* clade. Fungal Genetics and Biology. 41: 600-623.

O'Donnell, K., Kistler, H. C., Tacke, B. K. and Casper, H. H. 2000. Gene genealogies reveal global phylogeographic structure and reproductive isolation among lineages of *Fusarium graminearum*, the fungus causing wheat scab. Proceedings of the National Academy of Sciences (USA). 97: 7905-7910.

Osiewacz, H. D. 2002. Genes, mitochondria and aging in filamentous fungi. Ageing Research Reviews. (3): 425-442.

Parry, D.W., Jenkinson, P. and McLeod, L. 1995. Fusarium ear blight (scab) in small grain cereals- a review. Plant Pathology. 44: 207-238.

Paulsen, C. E. and K. S. Carroll. 2010. Orchestrating redox signaling networks through regulatory cysteine switches. ACS Chemical Biology. 5(1): 47-62.

Peraica, M., Radic, B., Lucic, A. and Pavlovic, M. 1999. Toxic effects of mycotoxins in humans. Bull. World Health Org. 77:754-766.

Peraza, L. and Hansberg, W. 2002. *Neurospora crassa* catalases, singlet oxygen and cell differentiation. Biological Chemistry. 383(3-4): 569-575.

Pirgozliev, S. R., Edwards, S. G., Hare, M. C., and Jenkinson, P. 2003. Strategies for the control of Fusarium head blight in cereals. European Journal of Plant Pathology. 109: 731-742.

Pritsch, C., Muehlbauer, G.J., Bushnell, W.R., Somers, D.A. and Vance C.P. 2000. Fungal development and induction of defence response genes during early infection of wheat spikes by *Fusarium graminearum*. Molecular Plant–Microbe Interactions. 12: 159-169.

Prudova, A., Bauman, Z., Braun, A., Vitvitsky, V., Lu, S. C. and Banerjee, R. 2006. S-adenosylmethionine stabilizes cystathionine beta-synthase and modulates redox capacity. Proceedings of the National Academy of Sciences (USA). 103(17): 6489-6494.

Proctor, R. H, Hohn, T. M and McCormick, S. P. 1995. Reduced virulence of *Gibberella zeae* caused by disruption of a trichothecene toxin biosynthetic gene. Molecular Plant– Microbe Interactions. 8: 593-601.

Pye, V. E., Christensen, C. E., Dyer, J. H., Arent, S. and Henriksen, A. 2010. Peroxisomal plant 3-ketoacyl-CoA thiolase structure and activity are regulated by a sensitive redox switch. The Journal of Biological Chemistry. 285(31): 24078-24088.

Rainwater, R., Parks, D., Anderson, M. E., Tegtmeyer, P. and Mann, K. 1995. Role of cysteine residues in regulation of p53 function. Molecular and Cellular Biology. 15(7): 3892-3903.

Ralser, M., Wamelink, M. M., Kowald, A., Gerisch, B., Heeren, G., Struys, E. A., Klipp, E., Jakobs, C., Breitenbach, M., Lehrach, H. and Krobitsch, S. 2007. Dynamic rerouting of the carbohydrate flux is key to counteracting oxidative stress. Journal of Biology. 6(4): 10.

Rampitsch, C., Bykova, N. V., McCallum, B., Beimcik, E. and Ens, W. 2006. Analysis of the wheat and *Puccinia triticina* (leaf rust) proteomes during a susceptible host-pathogen interaction. Proteomics. 6: 1897–1907.

Rampitsch, C., Subramaniam, R., Djuric-Ciganovic, S. and Bykova, N. V. 2010. The phosphoproteome of *Fusarium graminearum* at the onset of nitrogen starvation. Proteomics. 10(1): 124-40.

Rohde, J. R. and Cardenas, M. E. 2004. Nutrient signaling through TOR kinases controls gene expression and cellular differentiation in fungi. Current Topics in Microbiology and Immunology. 279: 53-72.

Rispail, N. and Di Pietro, A. 2009. *Fusarium oxysporum* Ste12 controls invasive growth and virulence downstream of the Fmk1 MAPK cascade. Molecular Plant–Microbe Interactions. 22(7):830-839.

Ritco-Vonsovici, M., Mouratou, B., Minard, P., Desmadril, M., Yon, J. M. and Andrieux, M. 1995. Role of the C-terminal helix in the folding and stability of yeast phosphoglycerate kinase. Biochemistry. 34: 833–841.

Rodaki, A., Young, T. and Brown, A. J. 2006. Effects of depleting the essential central metabolic enzyme fructose-1,6-bisphosphate aldolase on the growth and viability of *Candida albicans*: implications for antifungal drug target discovery. Eukaryotic Cell. 5(8): 1371-1377.

Rossignol, M., Peltier, J. B., Mock, H. P., Matros, A., Maldonado, A. M. and Jorrín, J. V. 2006. Plant proteome analysis: a 2004-2006 update. Proteomics. 6(20): 5529-5548.

Ruijter, G. J., Bax, M., Patel, H., Flitter, S. J., van de Vondervoort, P. J., de Vries, R. P., vanKuyk, P. A., and Visser, J. 2003. Eukaryotic Cell. 2: 690–698.

Sanchez, R., Riddle, M., Woo, J. and Momand, J. 2008. Prediction of reversibly oxidized protein cysteine thiols using protein structure properties. Protein Science. 17(3):473-481.

Santhanam, S., Venkatraman, A. and Ramakrishna, B. S. 2007. Impairment of mitochondrial acetoacetyl CoA thiolase activity in the colonic mucosa of patients with ulcerative colitis Gut. 56(11): 1543-1549.

Schaafsma, A. W., Tamburic-llincic, L., and Hooker, D. C. 2005. Effect of previous crop, tillage, field size, adjacent crop, and sampling direction on airborne propagules of *Gibberella zeae/ Fusarium graminearum*, Fusarium head blight severity, and deoxynivalenol accumulation in winter wheat. Canadian Journal of Plant Pathology. 27: 217-224.

Schroeder, H. W. and Christensen, J. J. 1963. Factors affecting resistance of wheat to scab caused by *Gibberella zeae*. Phytopathology. 53: 831–38.

Schwaller, M., Wilkinson, B. and Gilbert, H. F. 2003. Reduction-reoxidation cycles contribute to catalysis of disulfide isomerization by protein-disulfide isomerase. The Journal of Biological Chemistry. 278(9): 7154-7159.

Segmüller N., Kokkelink L., Giesbert S., Odinius D., van Kan J. and Tudzynski P. 2008. NADPH oxidases are involved in differentiation and pathogenicity in *Botrytis cinerea*. Molecular Plant– Microbe Interactions. 21(6): 808-19.

Seong, K., Hou, Z., Tracy, M., Kistler, H. C. and Xu, J. R. 2005. Random Insertional Mutagenesis Identifies Genes Associated with Virulence in the Wheat Scab Fungus *Fusarium graminearum*. Phytopathology. 95(7): 744-750.

Shaner, G. 2003. Epidemiology of fusarium head blight of small cereals in North America. In Fusarium Head Blight of Wheat and Barley, Leonard, K.J., Bushnell, W.R., Eds., The American Phytopathological Society: St. Paul, Minnesota, USA, 2003, Chapter 4, pp. 84-119.

Shenton, D. and Grant, C. M. 2003. Protein S-thiolation targets glycolysis and protein synthesis in response to oxidative stress in the yeast *Saccharomyces cerevisiae*. The Biochemical Journal. 374(2): 513-519.

Shipkova, P., Drexler, D. M., Langish, R., Smalley, J., Salyan, M. E. and Sanders, M. 2008. Application of ion trap technology to liquid chromatography/mass spectrometry quantitation of large peptides. Rapid Communications in Mass Spectrometry. 22(9): 1359-1366.

Solomon, P. S., Waters, O. D., Simmonds, J., Cooper, R. M. and Oliver, R. P. 2005. The *mak2* MAP kinase signal transduction pathway is required for pathogenicity in *Stagonospora nodorum.* Current Genetics. 48(1): 60-68.

Stack, R.W. 2003. History of Fusarium head blight with emphasis on North America. *In* Fusarium Head Blight of Wheat and Barley. Leonard, K. J., Bushnell, W. R., Eds. The American Phytopathological Society: St. Paul, Minnesota, USA, Chapter 1, pp. 1-34.

Strange, R. N., Majer, J. R. and Smith, H. 1974. The isolation and identification of choline and betaine as the two major components in anthers and wheat germ that stimulate *Fusarium graminearum* in vitro. Physiological Plant Pathology. 4: 277–290.

Sutton, J.C. 1982. Epidemiology of wheat head blight and maize ear rot caused by *Fusarium graminearum*. Canadian Journal of Plant Pathology. 4: 195-209.

Tag, A. G., Garifullina, G. F., Peplow, A. W., Ake, C. Jr, Phillips, T. D., Hohn, T. M. and Beremand, M. N. 2001. A novel regulatory gene, Tri10, controls trichothecene toxin production and gene expression. Applied and Environmental Microbiology. 67(11): 5294-52302.

Takemoto, D., Tanaka, A. and Scott, B. 2006. A p67Phox-like regulator is recruited to control hyphal branching in a fungal-grass mutualistic symbiosis. Plant Cell. 18: 2807–2821.

Takemoto, D., Tanaka, A. And Scott, B. 2007. NADPH oxidases in fungi: Diverse roles of reactive oxygen species in fungal cellular differentiation. Fungal Genetics and Biology. 44(11): 1065-1076.

Tavender, T. J., Springate, J. J. and Bulleid, N. J. 2010. Recycling of peroxiredoxin IV provides a novel pathway for disulphide formation in the endoplasmic reticulum. The EMBO Journal. 29(24): 4185-4197.

Taylor, R. D., Saparno, A., Blackwell, B., Anoop, V., Gleddie, S., Tinker, N. A. and Harris, L. J. 2008. Proteomic analyses of *Fusarium graminearum* grown under mycotoxin-inducing conditions. Proteomics. 8(11): 2256-2265.

Thines, E., Weber, R.W.S. and Talbot, N.J. 2000. MAP kinase and protein kinase A-dependent mobilization of triacyglycerol and glycogen during appressorium turgor generation by *Magnaporthe grisea*. Plant Cell. 12: 1703 – 1718.

Thon, M., Al-Abdallah, Q., Hortschansky, P. and Brakhage, A. 2007. The thioredoxin system of the filamentous fungus *Aspergillus nidulans*: Impact on development and oxidative stress response. The Journal of Biological Chemistry. 282(37): 27259–27269.

Trail, F. 2009. For blighted waves of grain: *Fusarium graminearum* in the postgenomics era. Plant Physiology. 149(1): 103-110.

Unwin, R. D., Griffiths, J. R., Leverentz, M. K., Grallert, A., Hagan, I. M. and Whetton, A. D. 2005. Multiple reaction monitoring to identify sites of protein phosphorylation with high sensitivity. Molecular and Cellular Proteomics. 4: 1134-1144.

Urban, M., Mott, E., Farley, T. and Hammond-Kosack, K. 2003. The *Fusarium graminearum* MAP1 gene is essential for pathogenicity and development of perithecia. Molecular Plant– Microbe Interactions. 4: 347–359.

Van Hemert, M. J., Steensma, H. Y., Van Heusden, G. P. 2001. 14-3-3 proteins: key regulators of cell division, signalling and apoptosis. Bioessays. 23(10):936-46.

Vignais, P. V. 2002. The superoxide-generating NADPH oxidase: structural aspects and activation mechanism. Cellular and Molecular Life Sciences. 59: 1428–1459.

Wang, Y. Z. and Miller, J. D. 1988. Effects of metabolites on wheat tissue in relation to Fusarium head blight resistance. Journal of Phytopathology. 122: 118-125.

Ward, T., Bielawski, J. P., Kistler, C. H., Sullivan, E. and O'Donnell, K. 2002. Ancestral polymorphism and adaptive evolution in the trichothecene mycotoxin gene cluster of phytopathogenic Fusarium. Proceedings of the National Academy of Sciences (USA). 99: 9278-9283.

Washburn, M. P., Wolters, D. and Yates, J. R. 2001. Large-scale analysis of the yeast proteome by multidimensional protein identification technology. Nature Biotechnology. 19(3): 242-247.

Weber, R.W.S., Wakley, G.E., Thines, E. and Talbot, N.J. 2001. The vacuole as central element of the lytic system and sink for lipid droplets in maturing appressoria of *Magnaporthe grisea*. Protoplasma. 216: 101 – 112.

Wei, J., Sun, J., Yu, W., Jones, A., Oeller, P., Keller, M., Woodnutt, G. and Short, J. M. 2005. Global proteome discovery using an online three-dimensional LC-MS/MS. Journal of Proteome Research. 4(3): 801-808.

Wienkoop, S. and Weckwerth, W. 2006. Relative and absolute quantitative shotgun proteomics: targeting low-abundance proteins in *Arabidopsis thaliana*. Journal of Experimental Botany. 57(7):1529-35.

Windels, C. E. 2000. Economic and social impacts of Fusarium head blight: changing farms and rural communities in the Northern Great Plains. Phytopathology. 90: 17-22.

Winterbourn, C. C. and Hampton, M. B. 2008. Thiol chemistry and specificity in redox signaling. Free Radical Biology & Medicine. 45(5): 549-561.

Wolf-Yadlin, A., Hautaniemi, S., Lauffenburger, D. A. and White, F. M. 2007. Multiple reaction monitoring for robust quantitative proteomic analysis of cellular signaling networks. Proceedings of the National Academy of Sciences (USA). 104: 5860-5865.

Wong, L. S. L., Tekauz, A., Leslie, D., Abramson, D., and McKenzie, R. I. H. 1992. Prevalence, distribution, and importance of Fusarium head blight in wheat in Manitoba. Canadian Journal of Plant Pathology. 14: 233-238.

Wood, Z. A., Poole, L. B. and Karplus, P. A. 2003. Peroxiredoxin evolution and the regulation of hydrogen peroxide signaling. Science. 300(5619): 650-653.

Xia, L., Yang, H., Xian, M. P. 2001. Cysteine-25 of adenylate kinase reacts with dithiothreitol to form an adduct upon aging of the enzyme. FEBS Letters. 507(2) : 169-173.

Xu, Y. G., and Chen, L. F. 1993. Wheat Scab: Theory and Practice on Control. Jiangsu Science and technology publishing House, Nanjing, China.

Xu, J. R. and Hamer, J. E. 1996. MAP kinase and cAMP signaling regulate infection structure formation and pathogenic growth in the rice blast fungus *Magnaporthe grisea*. Genes and Development. 10: 2696-2706.

Xu J. R., Peng Y. L., Dickman M. B., Sharon A. 2006. The dawn of fungal pathogen genomics. Annual review of Phytopathology. 44: 337-66.

Yano, H. and Kuroda, M. 2006. Disulfide proteome yield a detailed understanding of redox regulations: A model study of thioredoxin-linked reactions in seed germination. Proteomics. 6: 294-300.

Yazar, S. and Omurtag, G. 2008. Fumonisins, trichothecenes and zearalenone in cereals. International Journal of Molecular Sciences. 9: 2062-2090.

Ye, M. B., Xu, L. L., Xu, Y. G. and Zhu, B. 1990. Relationship between resistance of wheat to scab and activities of phenylalanine ammonia lyase and amount of chlorogenic acid. J. Nanjing Agriculture University. 13(3): 103–107.

Yun, C., Ding, L., Leng, Y., Zhu, H., Wen, A. and Yang, L. 2011. Determination of ipriflavone in human plasma by LC-MS and its application in a pharmacokinetic study. Biomedical Chromatography, 25: n/a. doi: 10.1002/bmc.1641

Zito, E., Melo, E. P., Yang, Y., Wahlander, A., Neubert, T. A. and Ron, D. 2010. Oxidative protein folding by an endoplasmic reticulum-localized peroxiredoxin. Molecular Cell. 40(5): 787-797.

Zheng, L., Campbell, M., Murphy, J., Lam, S. and Xu, J. R. 2000. The BMP1 gene is essential for pathogenicity in the gray mold fungus *Botrytis cinerea*. Molecular Plant– Microbe Interactions. 13(7): 724-732.

Zhu. W., Smith, J. W. and Huang, C. M. 2010. Mass spectrometry-based label-free quantitative proteomics. Journal of Biomedicine and Biotechnology. 2010 (840518): 1-6.