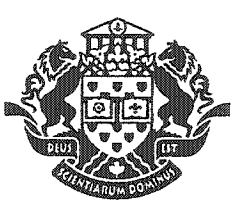


Université d'Ottawa · University of Ottawa

Université d'Ottawa · University of Ottawa

FACULTÉ DES ÉTUDES SUPÉRIEURES ET POSTDOCTORALES

FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

BORDELEAU, Christian

AUTEUR DE LA THÈSE - AUTHOR OF THESIS

M.Sc. (Biology)

GRADE - DEGREE

Biology

FACULTÉ, ÉCOLE, DÉPARTEMENT - FACULTY, SCHOOL, DEPARTMENT

TITRE DE LA THÈSE - TITLE OF THE THESIS

Characterization of Phosphorylation Changes and Protein Kinase Activities in Wheat Head Following Infection with the Fungal Pathogen Fusarium

D. Johnson

DIRECTEUR DE LA THÈSE - THESIS SUPERVISOR

EXAMINATEURS DE LA THÈSE - THESIS EXAMINERS

J.T. Arnason

M. Paulin-Levasseur

J. Vierula

J.-M. De Koninck, Ph.D.

LE DOYEN DE LA FACULTÉ DES ÉTUDES SUPÉRIEURES ET POSTDOCTORALES

SIGNATURE

DEAN OF THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

Characterization of phosphorylation changes and protein kinase activities in wheat head following infection with the fungal pathogen *Fusarium graminearum*

Christian Bordeleau

Thesis submitted to the
School of Graduate Studies and Research
Department of Biology
University of Ottawa
In partial fulfillment of the requirements for the
M. Sc. Degree in the
Ottawa-Carleton Institute for Biology

Ottawa, Ontario, Canada
August 7th, 2003

Your file Votre référence
ISBN: 0-612-90035-5
Our file Notre référence
ISBN: 0-612-90035-5

Résumé

La présence dans les récoltes de blé du champignon pathogène *Fusarium graminearum* pose de sérieux problèmes financiers et de santé au Canada. Le champignon cause des pertes significatives au niveau de la qualité et du rendement des récoltes, tout en produisant des mycotoxines qui survivent à la mouture. Les processus de transduction de signaux impliquant les kinases jouent un rôle prépondérant dans l'activation du système de défense de l'hôte.

Un projet de recherche, utilisant les hybridations de type western et les essais kinase, fut mis sur pied pour déterminer si la présence de *F. graminearum* causait un changement au niveau des kinases chez les cultivars Frontana (résistant) et Roblin (susceptible). Les résultats ont démontré que *F. graminearum* avait un effet modéré sur les niveaux de phosphorylation des acides aminés thréonine et tyrosine. De plus, certaines différences ont été observé entre les cultivars. Cependant, les essais kinases n'ont pas démontré une variation significative de l'activité enzymatique de type MAPK chez les cultivars.

Un homologue de la MAPK, *wmh-1* (wheat MAPK homologue-1) fut cloné, séquencé et fut démontré comme étant identique à la séquence de wck-1, que l'on peut trouver dans la banque de séquence publique. Des résultats préliminaires démontrent une augmentation du messager transcrit dans l'échantillon du Frontana traité au *Fusarium*. Les implications de ces résultats en sont discutées.

Abstract

The presence on wheat crops of the fungal pathogen *Fusarium graminearum* poses serious financial and health-related problems in Canada. The fungus causes a significant decrease in yield and quality of the crop, in addition to producing mycotoxins, which can survive the milling process. Signaling processes involving protein kinases are thought to be play a major role in the activation of a host defense response.

A research project employing western detection experiments and kinase assays was initiated to determine whether the presence of *F. graminearum* caused changes in the level of protein kinases in cultivars Frontana (resistant) and Roblin (susceptible). The results indicated that *F. graminearum* had modest effects on phosphorylation levels of threonine and tyrosine residues. Moreover, differences occurred between the resistant and susceptible cultivar. Kinase assays did not show any variation in activity in either cultivar.

A MAPK (wheat MAPK homologue-1) homologue was cloned, sequenced and shown to be identical to the *wck-1* sequence found in the public database. Preliminary results show an increase in the level of transcript in the *Fusarium*-treated Frontana sample. The implications of the results are discussed.

Remerciements

J'aimerais tout d'abord remercier mes superviseurs, les Drs Thérèse Ouellet et Doug Johnson pour leur support et leurs encouragements tout au long de ce périple. J'y ai acquis des outils supplémentaires pour affronter les obstacles de la vie.

Je voudrais également remercier mes collègues de laboratoires. Raymond Tropiano pour ses nombreuses attaques inoffensives à la langue de Molière (j'en ai ri jusqu'à en pleurer); Kasia Dadej pour ses suggestions et ses conseils que j'ai beaucoup appréciés; Anju Koul pour nos belles conversations enrichissantes aux sujets aussi divers que ces cultures qui nous séparent. Merci aussi, à tous les autres que j'ai côtoyés et qui ont su égayer les journées monotones qui font parfois partie de la recherche.

Il est également important de souligner le support et l'encouragement de ma famille ainsi que de mes amis. Sans eux, le travail aurait pris une toute autre dimension. Ils ont su me garder les deux pieds bien ancrés dans le présent. Leur aide m'a été très précieuse et je les en remercie.

Finalement, je voudrais remercier ma fée, celle qui m'a encouragé dans les moments les plus difficiles, et qui a continué de croire en cet homme qui aujourd'hui est heureux de pouvoir écrire ces dernières pensées.

TABLE OF CONTENTS

Résumé i

Abstract ii

Remerciements iii

Table of contents iv

List of Figures viii

List of Tables x

List of Abbreviations xi

Chapter 1 : Introduction

1.1 MAIN OBJECTIVES 1

1.2 BRIEF HISTORY OF FUSARIUM HEAD BLIGHT 2

1.3 EPIDEMIOLOGY OF *FUSARIUM GRAMINEARUM* 6

1.3.1 Description of the pathogen 6

1.3.2 Inoculum sources and production 7

1.3.3 Inoculum dispersal and colonization 10

1.4 THE EFFECTS OF FHB ON WHEAT 13

1.4.1 The involvement of mycotoxins 16

1.5 PLANT DEFENSE MECHANISMS 20

1.5.1 Different types of resistance 20

1.5.2 Breeding resistance 23

1.6 SIGNALLING AND PLANT DEFENSE 25

1.6.1 General aspects of plant defense 25

1.6.2 Detection methods of kinase activity and phosphorylation levels 29

1.6.3 The involvement of MAPKs in plant defense signalling 30

1.6.3.1 MAPK activation following abiotic stresses 35

1.6.3.2 MAPK activation following biotic stresses 36

1.6.4 The involvement of CDPKs in plant defense signalling 39

1.6.5 The involvement of other types of kinases in plant defense signalling 42

1.7 THE OBJECTIVES OF THIS STUDY 46

Chapter 2: Materials and methods

2.1 PLANT AND FUNGAL MATERIALS 50

2.2 INOCULATION WITH FUNGAL SPORES 50

2.3 PLANT PROTEIN EXTRACTION AND QUANTIFICATION 51

2.4 SDS GEL ELECTROPHORESIS 52

2.5 WESTERN ANALYSIS 53

2.5.1 Protein transfer *54*

2.5.2 Antibodies and detection methods *57*

2.6 IN-TUBE KINASE ASSAYS 58

2.7 IN-GEL KINASE ASSAYS 59

2.8 RNA AND DNA METHODS 60

2.8.1 RNA isolation *60*

2.8.2 DNA isolation *61*

2.8.3 Primer design for the amplification of MAPK by PCR and RT-PCR *62*

2.8.4 Gel purification of cDNA *69*

2.8.5 Ligation *70*

2.8.6 Transformation of DH5α by electroporation *70*

2.8.7 Plasmid isolation *71*

2.8.8 Radiolabelling of probes *72*

2.8.9 Northern hybridization analysis *72*

Chapter 3: Results

3.1 DETECTION OF PROTEIN BY WESTERN ANALYSIS 74

3.2 SPRAY-INOCULATED TIME-COURSE EXPERIMENTS 74

3.3 POINT-INOCULATED EXPERIMENTS 93

3.4 IN-TUBE KINASE ASSAYS 97

3.5 IN-GEL KINASE ASSAYS 103

3.6 ISOLATION OF *WMH-1* 106

3.7 NORTHERN HYBRIDIZATION 120

Chapter 4: Discussion **123**

4.1 SUMMARY OF RESULTS 124

4.2 WESTERN DETECTION EXPERIMENTS 126

4.2.1 Spray inoculations *126*

4.2.2 Point inoculations *134*

4.3 IN-TUBE KINASE ASSAYS 136

4.4 IN-GEL KINASE ASSAYS 138

4.5 NORTHERN HYBRIDIZATIONS 139

4.6 CONCLUSIONS 141

4.7 FUTURE STUDIES 142

References 146

Appendix A : Composition of Buffers 165

Appendix B: List of picked clones 168

Appendix C: Secondary antibody control 170

List of Figures

Figure 1: **A)** Distribution of *F. graminearum* in western Canada in 1995 and **B)** Distribution of *F. graminearum* in western Canada in 2001 *4*

Figure 2: *Fusarium graminearum* life cycle *8*

Figure 3: Fusarium head blight visual symptoms scale. *14*

Figure 4: Chemical structure of deoxynivalenol. *17*

Figure 5: General MAPK cascade. *32*

Figure 6: Summary of possible functions of CDPKs in a plant environment. *44*

Figure 7: Western analysis setup. *55*

Figure 8: Alignment of 7 MAPK sequences. *65*

Figure 9: Western detection experiment with *F. graminearum*-treated Roblin samples (anti-phospho-threonine). *76*

Figure 10: Western detection experiment with water- and *F. graminearum*-treated Roblin and Frontana samples (anti-phospho-threonine). *79*

Figure 11: Western detection experiment with *F. graminearum*-treated Roblin samples (anti-phospho-tyrosine). *82*

Figure 12: Western detection experiment with water- and *F. graminearum*-treated Roblin and Frontana samples (anti-phospho-tyrosine). *86*

Figure 13: Western detection experiment with *F. graminearum*-treated Roblin samples (anti-phospho-serine). *88*

Figure 14: Western detection experiment with water- and *F. graminearum*-treated Roblin and Frontana samples (anti-phospho-serine). *91*

Figure 15: Western detection experiment with point-inoculated water- and *F. graminearum*-treated Roblin and Frontana samples (anti-phospho-threonine and -tyrosine). *98*

Figure 16: In-tube kinase assay. *101*

Figure 17: In-tube kinase assay with positive control dilutions. *104*

Figure 18: In-gel kinase assay. *107*

Figure 19: PCR and RT-PCR on Roblin and Frontana samples. *110*

Figure 20: Alignment of nucleic acid sequences of *wck-1*, *wmh-1* to *–5*. *112*

Figure 21: Alignment of amino acid sequences of WCK-1, WMH-1 and WMH-5. *118*

Figure 22: Northern hybridization with *wmh-1* on Frontana and Roblin RNA samples. *121*

LIST OF TABLES

Table 1: Summary of known protein kinases and their targets *47*

Table 2: Summary of phosphorylation changes in the western detection experiments *94*

List of abbreviations

Ω	Ohms
°C	degrees Celsius
ATP	adenosine triphosphate
Avr	avrirulence
BSA	bovine serum albumin
Ca^{2+}	calcium
CaMK	calmodulin-dependent protein kinase
CCaMK	calcium and calmodulin-dependent protein kinase
cDNA	complementary DNA
CDPK	calcium-dependent protein kinase
CLD	calmodulin-like domain
cm	centimetre(s)
cpm	counts per minute
CRK	calcium-related protein kinase
cv	cultivar (s)
DEPC	diethyl pyrocarbonate
ddH_20	double distilled water
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleoside triphosphates
DON	deoxynivalenol
DTT	dithiothreitol

List of abbreviations (continued)

ECL	enhanced chemiluminescent
ECORC	eastern corn and oilseed research center
EDR-1	endhanced disease resistance 1
EDTA	ethylenediaminetetraacetic acid
ERE	elicitor response elements
FDK	*Fusarium*-damaged kernels
g	gram (s)
GSK	glycogen synthase kinase
h	hour (s)
H_2O_2	hydrogen peroxide
HCl	hydrogen chloride
IPTG	isopropylthio-β-D-galactoside
J	Joule (s)
kDa	kiloDalton (s)
kg	kilogram (s)
kV	kiloVolt(s)
L	Liter (s)
LZ	leucine zipper
M	molar
mm	milimetre (s)
MAPK	mitogen activated protein kinase
MAPKK	mitogen activated protein kinase kinase

List of abbreviations (continued)

MAPKKK	mitogen activated protein kinase kinase kinase
MBP	myelin basic protein
mg	miligram (s)
mg/kg	miligram (s) per kilogram (s)
mg/L	miligram (s) per liter (s)
mg/ml	miligram (s) per mililiter (s)
$MgCl_2$	magnesium dichloride
min	minute (s)
ml	mililiter (s)
mM	milimolar
$MnCl_2$	manganese dichloride
Na_3VO_4	sodium vanadate
NaCl	sodium chloride
NaF	sodium fluoride
NaI	sodium iodide
NaOAc	sodium acetate
$NaPP_i$	sodium pyrophosphate
NB-LRR	nucleotide binding-leucine rich region
ng	nanogram (s)
OAG	oleyl acetyl glycerol
PBF-2	Pr-10a binding factors

List of abbreviations (continued)

PCD/HR	programmed cell death/ hypersensitive response
PCR	polymerase chain reaction
PKC	protein kinase C
pM	picomolar
PMSF	phenylmethylsulfonyl fluoride
PR	pathogenesis-related
QTL	quantitative trait loci
R	resistance
RLK	receptor-like kinase
RNA	ribonucleic acid
rpm	revolution (s) per minute
RT	reverse transcriptase
RT-PCR	reverse transcriptase polymerase chain reaction
s	second (s)
SAR	systemic acquired resistance
SDS	sodium dodecyl sulfate
SIPK	Salicylic acid-induced protein kinase
TAE	Tris-acetate-EDTA electrophoresis buffer
TBS	Tris-buffered saline
TCA	trichloroacetic acid
TE	Tris-EDTA buffer

List of abbreviations (continued)

TIR	Toll/interleukin-1 receptor
tRNA	transfer RNA
µCi	microCurie (s)
µF	microFaraday (s)
µg	microgram (s)
µg/ml	microgram (s) per mililliter (s)
µg/µl	microgram (s) per microliter (s)
µl	microliter (s)
µM	micromolar
V	volt (s)
VIGS	virus-induced gene silencing
w/v	weight per volume
WIPK	wound-induced protein kinase
WMH-1	wheat MAPK homologue-1
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

Chapter 1
Introduction

1.1 Main objectives

Fusarium head blight is a fungal disease of wheat (*Triticum aestivum*, L.) caused by several species of the genus *Fusarium*. Two species are considered to play major roles in spreading this disease throughout North America: *Fusarium graminearum* Schwabe (teleomorph = *Gibberella zea* (Schw.: Fr) Petch) and *F. culmorum*. In Canada, the occurrence of periodic epidemics of Fusarium head blight results in major yield and quality losses for the producers. Cultivars of wheat showing different levels and types of resistance have been identified through worldwide screening efforts. In addition breeding programs have attempted to transfer resistance to commercial wheat cultivars. Since wheat is a polyploid (*Triticum aestivum*, 6X = 3N), this offers a significant challenge to the breeder.

Understanding the mechanisms that plants employ to defend themselves against pathogens is a crucial area of research. Protein kinases have been identified in a variety of plant hosts as the key regulatory proteins that participate in signal transduction following biotic and abiotic stresses, including infection. The main objectives of this thesis are to look at the involvement of protein kinases in wheat defense mechanisms after point- and spray inoculations with a fungal pathogen, such as *Fusarium graminearum* in two different wheat cultivars: Roblin (susceptible variety) and Frontana (resistant variety), and to identify those

kinases that respond to infection and thus are possible candidates for kinases involved in plant defense.

1.2 Brief history of Fusarium head blight

Fungal pathogens such as *F. graminearum*, *F. culmorum* and *F. avenaceum* ((Corda: Fr.) Sacc.) have developed an intricate relationship with their cereal hosts, causing heavy damage from Fusarium head blight (FHB) (Atanassov *et al.* 1994). Early surveys conducted by the Plant Disease Survey in the United States in 1917, 1918 and 1919 showed that 31 of 40 states reported FHB in cereals (Atanasoff, 1920). *Fusarium graminearum* was first identified in Manitoba in 1923, yet serious outbreaks were only reported in early the 1980s (Clear and Patrick, 2002). Severe epidemics threatened crops throughout North America. The problem has arisen in the eastern and mid-western regions of the United States. These epidemics have caused extensive damage to wheat and barley crops in four successive years, from 1993 to 1996. Yield losses in the state of Minnesota have been estimated at 33% in 1993, 18% in 1994 and 8% in 1995 (Dill-Macky and Jones, 1997). In 1980, a severe epidemic of Fusarium head blight of wheat in Eastern Ontario caused farmers in the area to lose millions of dollars in lost crops (Sutton, 1982). Major epidemics reduce greatly the viability of the infected crop and in turn reduce profit margins for producers. Mycotoxin analysis shows that this fungus has continued to spread in western Canada, where high levels of deoxynivalenol and its precursors were found (Abramson *et al.* 1987). These mycotoxins present a major health hazard to

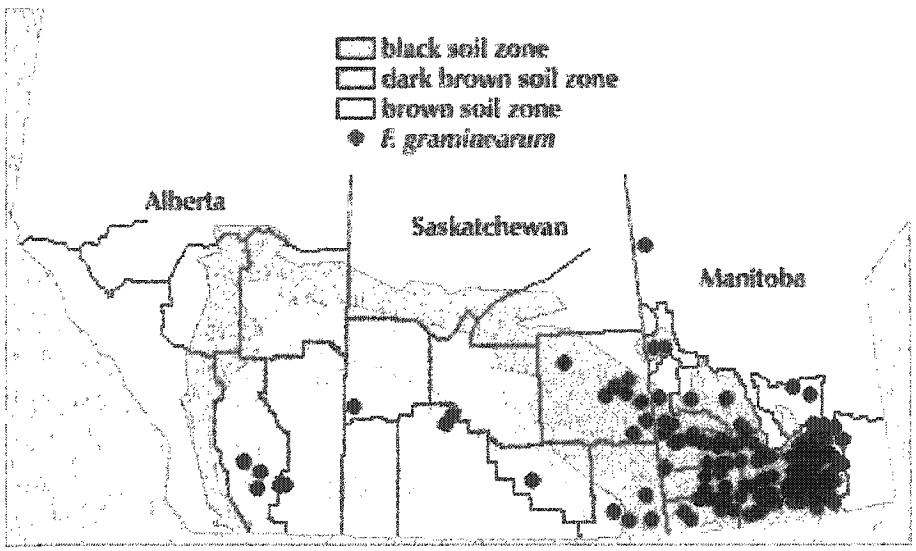
consumers as well as livestock. Western Canada has seen an increase in the number of crops infected with *F. graminearum*, *F. culmorum* and *F. avenaceum*.

Since the early 1980s, surveys have been conducted by the Grain Research Laboratory in western Canada in order to monitor the *F. graminearum* populations. These surveys indicate the ever-growing impact of *F. graminearum* on Canadian crops (Clear and Patrick, 2002). This devastating impact is starting to reach northern British Columbia and threatens to continue its infectious spread. Figure 1A shows the areas where *F. graminearum* infected crops were reported in 1995. The extent of the damage is mostly found in Manitoba due to its higher average rainfall and higher mean temperature in July (Clear and Patrick, 2002).

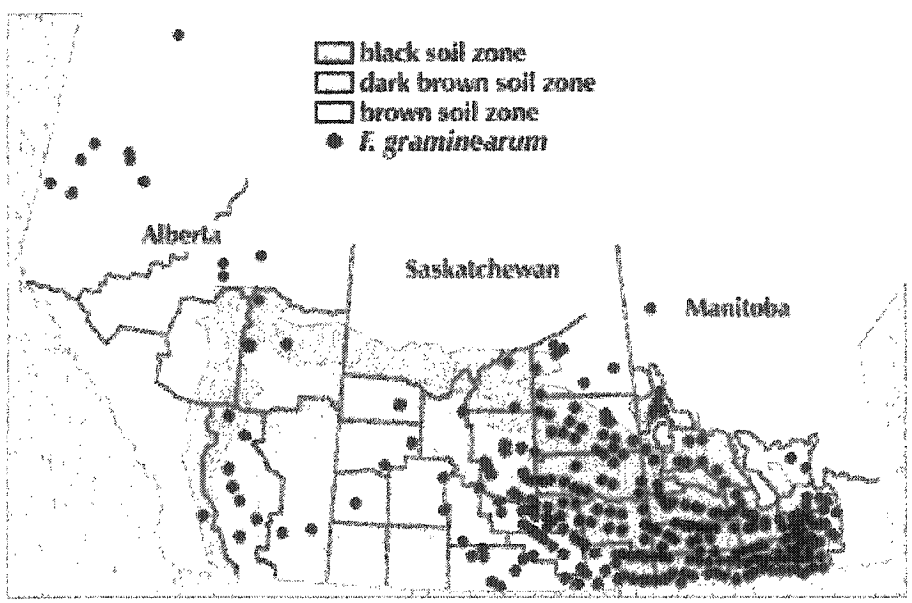
Six years later, the same survey was conducted and showed a greater distribution of *F. graminearum* throughout western Canada (Figure 1B). Its distribution had spread through northern Alberta and into northern British Columbia. The majority of cultivated grain crops in Manitoba as well as in eastern Saskatchewan, showed signs of Fusarium head blight. The Grain Research Laboratory surveys have also monitored the percentage of *Fusarium*-damaged wheat kernels (FDKs). In 1996, the percentage of FDK found in Manitoba wheat was 34.7% while in 2001, the percentage of FDK in the same province had risen to 69%. In the same time span, the FDK percentage in Saskatchewan went from 0.4% to 8%, while Alberta showed an increase from 0.02% to 1% (Clear and Patrick, CGC, 2002). In addition, the geographical distribution of the fungus is related to temperature requirements. In hotter

Figure 1: **A)** Distribution *of F. graminearum* in 1995 **B)** Distribution of *F. graminearum* in 2001 based on the presence of FDK in the seed surveys. Survey conducted by the Canadian Grain Commission (Clear and Patrick,CGC,2002).

A) *F. gramiearum* sites in 1995



B) *F.graminearum* sites in 2001



regions, *F. graminearum* is generally regarded as the most important species causing FHB (reviewed by Parry *et al.* 1995). The high dispersal activity of this fungus has mobilized efforts in trying to understand the pathogen and its epidemiology.

1.3 Epidemiology of Fusarium graminearum

1.3.1 Description of the pathogen

Within the genus *Fusarium*, many species have been recognized as responsible for small grain cereal diseases. *Fusarium graminearum*, the anamorph or conidial stage of *Gibberella zeae*, is the principal head blight and ear rot pathogen in Canada (reviewed by Sutton, 1982). In North America, the population of *F. graminearum* can be subdivided into two groups: Group I and Group II (Francis and Burgess, 1977; Cook, 1981). Group I populations are traditionally regarded as soil borne fungi, where they exist as colonizers of plant residues and living plant parts. These isolates are normally associated with diseased crowns and do not form perithecia in culture and only rarely in nature (Sutton, 1982). Group II populations are generally primary or secondary colonizers of aerial plant parts (Burgess, 1981). These isolates readily form perithecia and normally are associated with diseases of aerial plant parts (reviewed by Sutton, 1982). The conidiophores are at first simple monophialides on the hyphae, but will later branch off and proliferate. The macroconidia are slender, sickle-shaped with curved apical and pedicellate cells and are mostly 5-6 septate. In order to understand the complexity of the problem, it is helpful to

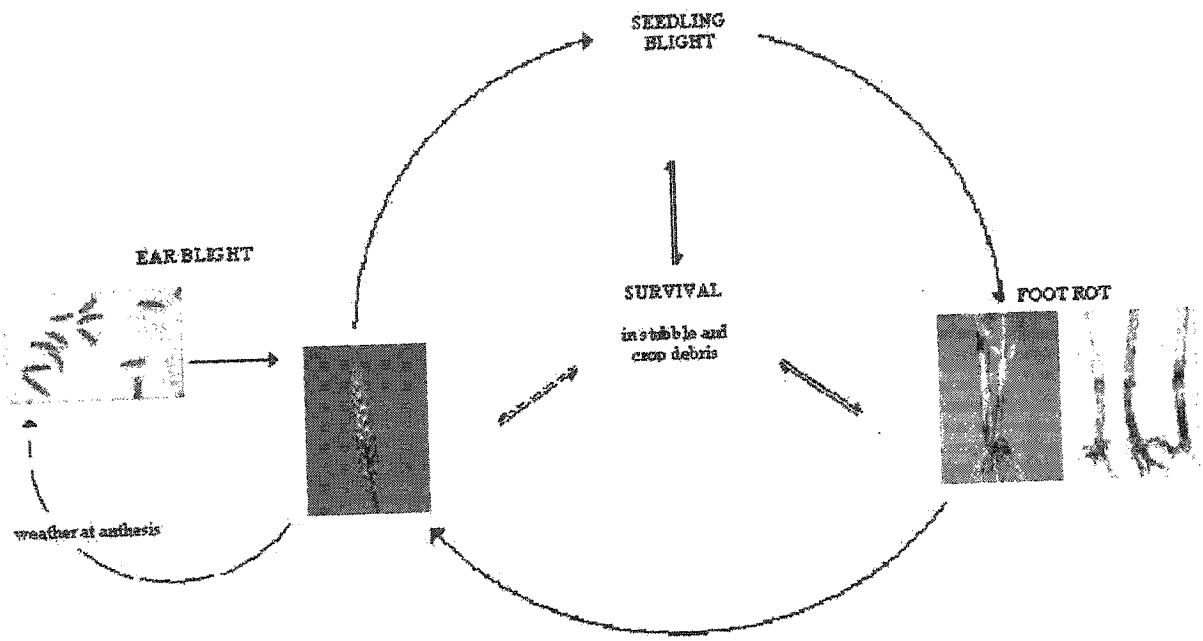
examine the growth and dispersal of *Fusarium*, and the disease cycle on small grain cereals. The life cycle of Fusarium graminearum is presented in Figure 2.

1.3.2 Inoculum sources and production

Common to all *Fusarium*-related problems is the presence of *F. graminearum* in soil, weeds, alternative hosts etc., which can function as reservoirs for the fungus. The *Fusarium* inoculum can survive as either saprophytic mycelium or thick-walled resting spores (chlamydospores) (reviewed by Parry *et al.* 1995). Old straw and pieces of stems of last year's crop, which had been heavily infected with the disease, were often found to show large pinkish areas indicative of *F. graminearum* conidia (Atanasoff, 1920). It was shown by Wearing and Burgess (1977) that fungus recovered from wheat-field soil could be grown on minute pieces of organic debris. Contaminated grain can also provide a primary source of inoculum for the development of seedling blight and foot rot (reviewed by Parry *et al.* 1995). A study carried out in Minnesota showed that of the 80% of maize stalks, which were infected with *F. graminearum* in the fall, 60% were still infected the following spring (Windels and Kommedahl, 1974). Recent studies have shown that continued wheat cropping or crop rotation between corn and wheat increases the inoculum source of *Fusarium*, which in turn increases the chance of ear or head blight epidemics (reviewed by Parry *et al.* 1995). As mentioned above, the inoculum takes the form of conidia, chlamydospores or hyphal filaments. However for *F.*

Figure 2: Life cycle of the fungal pathogen, Fusarium graminearum.

Taken from http://www.csl.gov.uk/science/organ/environ/entom/fusarium/lifecycle.cfm.


SEEDLING BLIGHT
EAR BLIGHT
SURVIVAL
in stubble and crop debris
FOOT ROT
weather at anthesis

graminearum the ascospores are believed to be the primary source of inoculum, through the sexual stage of *Gibberella zeae* (reviewed by Stack, 1999). Inoculum production is usually associated with warm and moist weather. When isolates of *G. zeae* were cultured on carnation leaf discs mounted on water agar in a temperature gradient plate ranging from 16°C to 31°C, perithecia formation increased as the temperature increased, at least to 29°C (Tschanz *et al.* 1976). Cook and Christen (1976) showed that *F. graminearum* grew optimally at -10 to -20 bars at temperatures ranging from 20°C to 30°C. It was also shown that on agar media adjusted to different water potentials, the maximum production of ascospores occurred at -15 bars for Group I *F. graminearum*. However, Group II *F. graminearum* isolates had maximum sporulation at -1.4 to -3.0 bars (Sung and Cook, 1980). Although different species may have different thermal requirements, one premise remains the same; a warm and relatively moist environment is required for the production of *Fusarium* inoculum.

1.3.3 Inoculum dispersal and colonization

The mode of dispersal of the inoculum presents several aspects. Recent field observations show that *G. zeae* ascospores were recovered mostly at night (20:00-08:00 hours) with a 1.5 log fewer spores in the late afternoon (16:00-20:00 hours) and morning (08:00-12:00 hours) (Fernando *et al.* 2000).

Wind dispersal and rain splashing are regarded as the principal modes for propagation of the spores. In an early study, Atanasoff (1920) observed that

incidence of ear blight in a rye field was increased in exposed areas in the direction of the prevailing wind. In areas sheltered by wind, ear blight was less common.

Although high humidity is necessary for initial release of ascospores, it would appear that dry periods are necessary for forceful discharge of ascospores into the air (reviewed by Parry *et al.* 1995). Afterwards, the importance of rain in the dispersal is paramount. A dramatic effect was observed in wheat crops receiving overhead irrigation. Strausbaugh and Maloy (1986) showed that 89% of wheat heads, which were irrigated, were infected with *F. avenaceum*, *F. culmorum*, *F. graminearum* and *Monographella nivale* while no heads were infected in non-irrigated crops. Rain-splash as well as irrigation contributed to the dispersal of spores. It was estimated that a single drop of water, 5mm in diameter, falling on a spore suspension of 1.6×10^7 spores/ ml of water, at a 0.1 mm depth, produced 2000 splash droplets carrying spores over a distance of over 75 cm horizontally (Gregory *et al.* 1959). In addition, the timing of the rainfall seems to be critical for the development of the head blight epidemic. The incidence of spikelet infection and the total rainfall during the period June 11 to July 11, during which the heads were at anthesis, was strongly correlated (Snijders, 1990).

Insects appear to serve as vectors of *F. graminearum* in the fields. Windels *et al.* (1976) showed that adult picnic beetles (*Glischrochilus quadrisignatus*) act as vectors for 7 different *Fusarium* species including *F. graminearum*, due to their high activity among plant and debris (reviewed by

Sutton, 1982). When birds such as red-winged blackbirds or starlings feed on maize ears when the kernels are at the milk stage of development, the birds shred the husk, puncture the kernel and remove the pericarp contents. This damage predisposes the maize to pink ear rot (reviewed by Sutton, 1982). Thus, activities from birds and insects as well as environmental conditions influence the extent of *F. graminearum* spore infection on small grain cereals and corn.

Once *F. graminearum* infects the plant, several factors determine whether the disease will develop. The general consensus is that wheat heads are most susceptible during the flowering stage (Atanasoff, 1920; reviewed by Sutton, 1982). This hypothesis was confirmed when Atanasoff (1920) inoculated wheat heads with spores from *F. graminearum* at various growth stages ranging from "just heading" to "late-dough stage". He found that disease was most severe in plants that had been inoculated during the anthesis stage. This led to the suggestion that anthers promote the initial infection. Inoculating spores onto normal spikelets compared to spikelets in which the anthers had been removed (emasculated) produced convincing evidence. Emasculated spikelets rarely became infected while infection was frequent in non-emasculated spikelets (Strange and Smith, 1971). It was also shown by histological studies that infection with *F. graminearum* frequently began in retained anthers before spreading to other parts of the spikelet (Pugh *et al.* 1933). Powerful infection stimulants such as pollen, choline, betaine and quaternary ammonium compounds, have been recovered from wheat anthers (Strange and Smith, 1971). Pieces of filter paper soaked with anther extract, choline or betaine

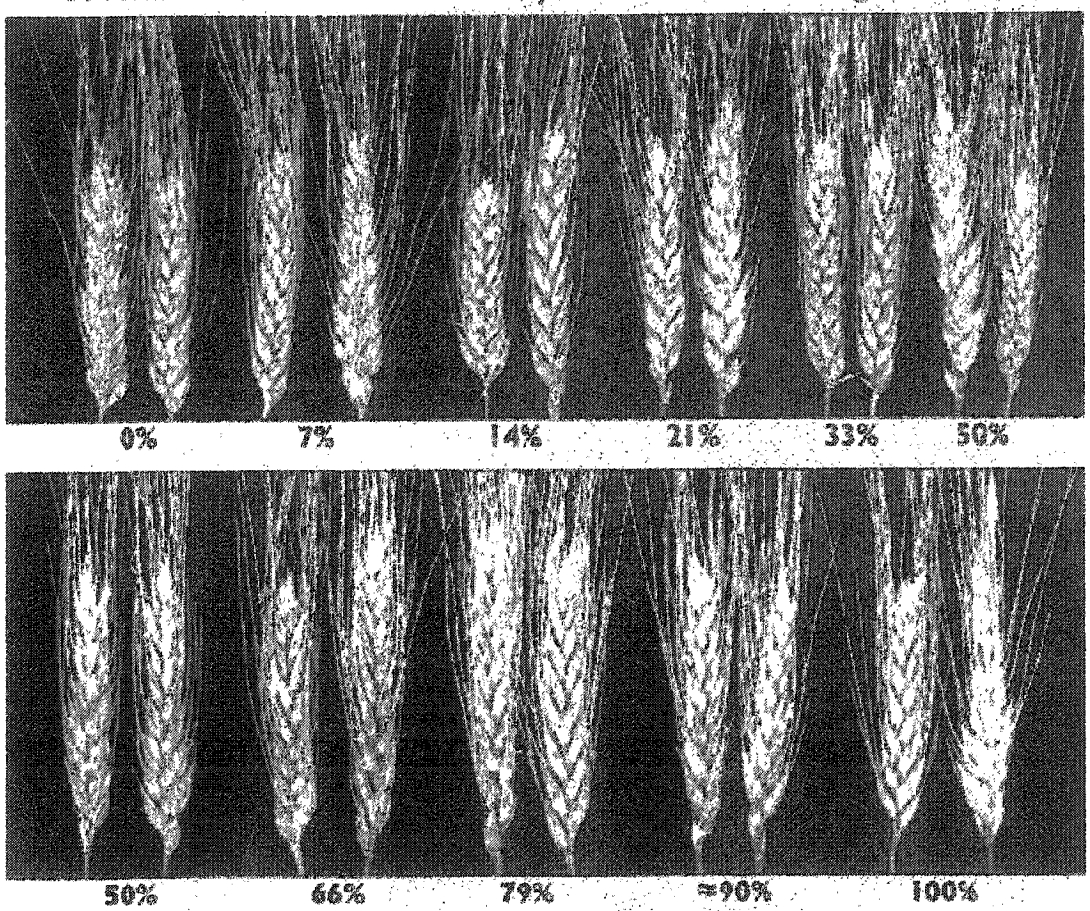
stimulated the infection of the florets of winter wheat with *F. graminearum* (Strange *et al.* 1978) thus confirming their importance in the infection process.

1.4 The effects of FHB on wheat

F. graminearum attacks a wide range of host plants, but mainly targets small grain cereals and corn. Once *F. graminearum* has infected a susceptible species such as wheat, the fungus causes biochemical and structural changes in the kernels. Early reports defined the symptoms of infection. Kernels from heads infected shortly after anthesis showed small size, had a pale greenish colour, were badly shrunken, lacked firmness and showed reduced weight. As a general rule the earlier the kernel is infected with *F. graminearum*, the greater the observed damage (Atanasoff, 1920). The earliest infections generally kill the floret and no kernel will develop. However, florets infected at a later stage may bear partially developed kernels (said to be "tombstone" kernels) (reviewed by Stack, 1999). The symptoms of infection leading to blight are also very well understood (Figure 3). The first signs of infection are slightly brown and water-soaked spots, 2-3 mm in length found on the glumes. The veins appear more water-soaked and have a much darker olive-green colour. The points of attachment between the glumes and the rachis are also water-soaked (Atanasoff, 1920). More recent experiments have defined the effects of *F. graminearum* infection on yield e.g., a 38-51.7% loss was caused in winter rye (Miedaner *et al.* 1993). It was also shown that *F. culmorum* reduced 1000-kernel weight in 10

Figure 3: Visual scale of Fusarium head blight symptoms on wheat heads, ranging from 0 – 100% infection. Taken from http://www.smallgrains.org/techfile/fusarium.htm

A Visual Scale to Estimate Severity of Fusarium Head Blight in Wheat

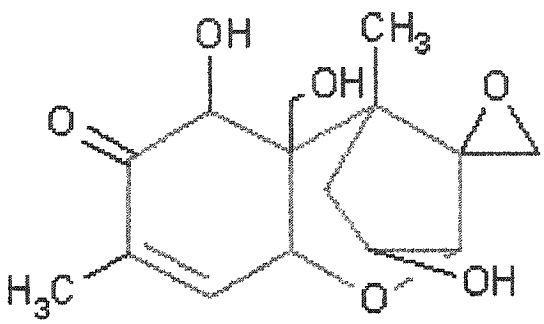


winter wheat genotypes by 2.8-22.4% (Snijders and Perkowski, 1990). According to Bechtel *et al.* (1985), the infection of wheat grain by *F. graminearum* destroys the starch granules, storage proteins and cell walls thus reducing seed germination and vigour. In addition to being a source of contamination, *Fusarium* has also the potential of producing mycotoxins, such as deoxynivalenol (DON) and zearalenone. These mycotoxins pose a serious health threat.

1.4.1 The involvement of mycotoxins

Deoxynivalenol (Figure 4), a member of the trichothecene family, is produced by a number of fungal species such as *Fusarium*, *Myrothecium*, *Trichoderma*, *Cephalosporium*, *Verticimonosporium* and *Stachybotrys* (Ueno, 1977). Trichothecenes constitute a large family of sesquiterpene epoxides, which inhibit eukaryotic protein synthesis (Desjardins and Hohn, 1997). Within this family are several important mammalian toxins including nivalenol, deoxynivalenol, T-2 toxin, diacetylnivalenol and its derivatives (Ueno, 1977). Experiments using pure trichothecenes at low dosages have demonstrated alarming toxicity to animals including humans. The observed symptoms included anemia, immunosuppression, haemorrhaging, diarrhoea, abortion, necrotic angina, destruction of bone marrow, inflammation, emesis and feed refusal. Refusal of feed has also been attributed to zearalenone, an estrogenic analog produced by *Fusarium* spp in infected corn (Kotsonis *et al.* 1975). Trichothecenes are acutely toxic to the liver and kidney of experimental animals.

Figure 4: Chemical structure of deoxynivalenol produced by *Fusarium* species. Courtesy of mycoherbicide.net/CHEMWARFARE/ chemical_ warfare.htm


OH
CH3
OH
O
O
H3C
O
OH

For toxicity use, lethal doses for deoxynivalenol have been measured in many experimental systems such as mice (males)(70 mg/kg), ducklings (10-day-old)(27 mg/kg) and rabbit reticulocytes (2.0 ug/ml of whole cell) (Vesonder and Hesseltine, 1981). In addition, a fatal disease known as alimentary toxic aleukia has been observed in human populations exposed to over-wintered grains containing T-2 toxins (Desjardins and Hohn, 1997; Ueno, 1977).

These mycotoxins are highly toxic at the subcellular, cellular and organic system levels. They readily penetrate lipid bilayers allowing easy access to cellular organelles and processes within the cell, e.g., DNA synthesis (reviewed by Rousseaux, 1988). Inhibition of protein synthesis may occur at three levels: inhibition of initiation, inhibition of elongation and inhibition of termination; or inhibition of all three steps through the inhibition of peptidyl transferase (reviewed by Rousseaux, 1988). Wang and Miller (1988) observed that a DON concentration of 10^{-6} M (0.3 mg/L) strongly inhibited coleoptile growth in some susceptible wheat cultivars while wheat cultivars resistant to FHB were 10-100 times more tolerant. This toxicity can be disrupted through the mutation of the fungal trichodiene synthase gene (*Tri5*). The *Tri5* gene encodes the enzyme trichodiene synthase, which catalyzes the first unique step of the trichothecene pathway (Hohn *et al.* 1995). The loss of function of this gene showed a decrease in virulence of *G. zeae* on wheat (Proctor *et al.* 1995). Trichothecene-nonproducing mutants (*Tri5* $^{-}$) were less virulent than the trichothecene-producing (*Tri5* $^{+}$) parent in their ability to cause head blight. Although trichothecene-nonproducing mutants did colonize wheat heads, the infected heads showed

fewer disease symptoms such as head bleaching, seed weight, seed viability, and trichothecene contamination (Desjardins *et al.* 1996). Atanossov *et al.* (1994) looked at the correlation coefficients for grain weight reduction and mycotoxin concentrations in 15 wheat genotypes ranging from highly resistant to highly susceptible infected with 6 different strains of *F. graminearum* and concluded that DON and related trichothecenes play a role as virulence factors in disease development. This work was corroborated by Perkowski *et al* (1990) who observed a correlation between the amount of DON metabolites and the percentage of FDK present. Thus, the susceptibility of wheat cultivars to fungal infections will dictate the extent of the damage caused by the infection.

1.5 Plant resistance mechanisms

1.5.1 Different types of resistance

Plants have developed a range of mechanisms in order to respond to different environmental stimuli appropriately. Plants have yet to develop the 'fight or flight' response, obliging them to fight in order to survive. Most plants offer general resistance to pathogens. Passive protection against non-specific pathogens is provided by structural barriers such as waxy cuticle and preformed reservoirs of anti-microbial compounds (reviewed by Hutcheson, 1998; reviewed by Dangl and Jones, 2001). Also included in the passive defense mechanisms are: plant height, where dwarfness increases natural disease occurrence; the presence of awns, which increases disease severity; spikelet density, which increases natural infection and grain membrane thickness, which increases with

the maturation process (Cook, 1981; Pugh, 1933). A dwarf genotype provides a shorter distance between the leaves and the soil, allowing a bigger rain splash effect. The presence of awns helps to accumulate airborne spores by increasing the head surface (Miedaner, 1997). The membrane thickness antifungal property was observed by Pugh *et al* (1933) where they found that as grain matured, they became more resistant to hyphae penetration by *G.zeae* (*F. graminearum*). This resistance was correlated with the thickness of the semi-permeable membranes of the grain. Therefore, as the membrane increased in thickness during the maturing process, so did the resistance to fungal invasion. Once the passive defense mechanisms have been circumvented, plants have a second line of defense including induced (or active) cellular defense mechanisms. Schroeder and Christensen (1963) described two types of resistance mechanisms for resistance to wheat scab caused by *F. graminearum*: I) resistance to initial infection and II) resistance to the spread of the infection. Type I resistance is usually localized to the cells in contact with the pathogen or close to a pathogenic structure with the outcome frequently being programmed cell death / hypersensitive response (PCD/HR) (reviewed by Hutcheson, 1998). Yet, Schroeder and Christensen (1963), failed to find any histological features associated with such resistance and assumed that such resistance was physiological.

Type II resistance, also termed "secondary responses" bears some similarity with a defense mechanism observed in other plant/pathogen interaction which is induced in the adjacent cells surrounding the initial site of infection

(Hutcheson, 1998). Schroeder and Christensen (1963) showed significant differences in varietal reactions towards spray inoculations and injection inoculations. When plants were spray inoculated with a conidial suspension of *F. graminearum*, varieties Thatcher and Mindum were most susceptible to infection while Frontana and Erythrospermum 3086-30M were most resistant to infection. When plants were inoculated by injection into the medial spikelets, marked differences were observed. Thatcher and Mindum varieties were most susceptible to pathogen spread while Lee and Rival were most resistant to pathogen spread. Lee, for example, was quite susceptible to the initial pathogen infection but demonstrated good resistance to hyphal spread in the spike. Therefore, two different and independent defense mechanisms may be involved in plant responses to pathogen infection.

A third type of resistance (Type III) was first observed by Miller and Arnison (1986) who found that resistant cereal lines contained lower concentrations of DON compared to susceptible cultivars. In their study, 20 cultivars of spring cereals were infected with a single strain of *F. graminearum*. The results indicated that disease-resistant cultivars of wheat such as Frontana had lower concentrations of DON as well as a smaller fungal biomass than susceptible cultivars. In addition, there was proportionally less DON in Frontana relative to susceptible cultivars than the biomass measurement indicated. This suggested that resistant cultivars have factors that prevent synthesis and /or promote the degradation of DON. It was later shown that the rate of decline of

DON levels was also cultivar specific (Miller and Arnison, 1986). Thus in resistant cultivars, type III mechanisms result in faster rate of DON degradation.

Wang and Miller (1988) found that resistant wheat cultivars were 10-1000 times more tolerant to DON and 3-acetyl-DON than susceptible cultivars. They suggested that there is another mechanism of resistance of wheat *to F. graminearum*, Type IV. Hence, this type of resistance would confer endurance to high levels of DON.

Finally, tolerance has been mentioned as a fifth mechanism of resistance. However, this term can be summarized as the ability of the plant to endure effects of parasitic infection, if occurred in a plant of same and/or similar species would cause a greater loss of yield and growth (Mesterhazy, 1995).

Thus, in summary, type I resistance gives resistance to the initial infection, while type II resistance confers resistance to the infectious spread of the hyphae. Type III resistance promotes the degradation of DON at a faster rate than susceptible cultivars in contrast to type IV resistance which confers a higher endurance to increased levels of DON. Finally, type V resistance confers tolerance to parasitic infection.

1.5.2 Breeding for resistance

In order to develop more resistant cultivars, breeding programs around the world have been initiated. These programs have produced resistant cultivars such as Frontana and Sumai3. The complexity of the resistance mechanisms and the hexaploid nature of wheat make breeding for resistance difficult and time

consuming. The number and types of resistant mechanisms mentioned above suggest that resistance is polygenic and may function best in certain genetic backgrounds (Mesterhazy, 1995).

Despite all these obstacles practical solutions have been developed and it is now possible to have a structural ideotype that reduces infection: plant height of approximately 90-100cm, an awnless plant, distance between flag leaf and ear should be a minimum of 15cm and the head should not be too dense (Mesterhazy, 1995). Yet control of these physical attributes is only a first step in breeding for possible disease control genes.

Genetic variation in wheat cultivars has been reported as early as 1929 (Schroeder and Christensen, 1963). Resistant germplasm can be divided into three gene pools: winter wheats from Eastern Europe (Praag 8, Novokrumka 0102), spring wheats from China and Japan (Ning 8343, Wuhan, Nobeoka bozu Komugi, Sumei 3, Pekin 8) and spring wheats from Brazil (FT 83-326, Frontana) (Snijders, 1990; Mesterhazy, 1995). Resistance has proven to be very stable throughout the years, allowing them to be valuable parents when crossed with other resistant genotypes. The stability of a resistance is dependent upon resistance and/or susceptibility levels. Resistant genotypes remain healthy under epidemic conditions or have only slight disease symptoms (Mesterhazy, 1995). Even though, there is a high genotypic variance between cultivars, a 'genotype-by-environment'-type of interaction plays an important role in the wheat / *F. graminearum* relationship (Miedaner, 1997). An experiment spanning 6 years showed correlation coefficients between two years ranging from 0.19 to 0.81 for

head blight rating. In addition, this variation was important in highly resistant and highly stable cultivars (Mesterhazy, 1995). Therefore, environmental parameters play such an important role as to render the analysis of the results very difficult (Mesterhazy, 1997).

Experiments reported by Bai *et al.* (1993) suggested that 1 to 3 genes were responsible for most of the resistance to *F. graminearum* head blight in Chinese materials. The resistance genes were associated with 2-5 different wheat chromosomes. For example, in Sumai3, the resistance genes were on chromosomes 2A, 5A, 1B, 6D and 7D chromosomes (Bai and Shaner, 1994).

1.6 Signalling and plant defense

1.6.1 General aspects of plant defense

The plant kingdom contains thousands of resistance (*R*) genes, with specific responses to particular viral, bacterial, fungal pathogens. The pathogen genes whose expression causes a defense response in the plant host containing the appropriate *R* gene are called avirulence (*Avr*) genes. The gene-for-gene interaction hypothesis, first developed by Flor in 1947, states that plants contain single dominant *R* genes that specifically recognize pathogens that contain the complementary *Avr* genes (reviewed by Staskawicz, 2001). Flor used rust resistance in flax as a model system to elaborate four areas of research: "new approaches in the studies of new races, mutations for rust reaction in the host and pathogenicity in the parasite, the evaluation of epidemiological data, and the nature of resistance" (Flor, 1947). In one plant, we can find many *R* genes, while

a pathogen may have many *Avr* genes. A strong resistance response occurs when an *Avr* gene and a corresponding *R* gene are expressed by both pathogen and host, respectively (Keen, 1990). A hallmark response for a gene-for-gene interaction is the activation of a hypersensitive response (HR), in which programmed cell death occurs in the immediate vicinity of the infection (reviewed by Jones and Dangl, 1996). Other features of the response include induced synthesis of antimicrobial compounds (phytoalexins) (Lodge *et al.* 1993), calcium influx into the cell, production of reactive oxygen intermediates (reviewed by Dangl and Jones, 2001) synthesis of proteins that can be harmful to the pathogen (such as glucanases and chitinases; also known as pathogenesis-related proteins) (Cordero *et al.* 1994) and reinforcement of plant cell walls (Levine *et al.* 1994).

In the past decade, tremendous effort has been made to identify these *R* genes. Functional *R* genes isolated thus far confer resistance to bacterial, viral, fungal, oomycete, nematode and even insect pathogens (reviewed by Dangl and Jones, 2001). The largest class of *R* genes encodes proteins containing "nucleotide binding region plus leucine-rich repeat" motifs (NB-LRR)(reviewed by Bent, 1996). LRRs are serial repeats of a 24 amino acid motif, usually containing leucines or other hydrophobic residues (Kobe and Deisenhofer, 1994). LRRs are found in diverse proteins and usually function as sites for protein-protein interactions, peptide-ligand binding and protein-carbohydrate interaction (reviewed by Kajava, 1998). In the gene- for-gene hypothesis the LRR domain of

the *R* gene product serves as the binding domain for a ligand produced due to the *Avr* gene activity (Bent, 1996).

In addition, each *R* gene of this class contains a conserved nucleotide-binding site. These domains occur in proteins with ATP or GTP binding activity such as ATP synthase subunits, ras proteins, ribosomal elongation factors and adenylate kinases (Saraste *et al.* 1990). The presence of the highly conserved NB domain in *R* genes suggests that the binding of nucleoside triphosphates is essential for the proper functioning of these proteins.

Within the NB-LRR subgroup, further subgroups can be ascertained; those contain the leucine zipper (LZ) motif or those similar to the Toll/interleukin-1 receptor (TIR). Leucine zipper sequences facilitate protein-protein interactions by introducing the formation of coiled-coil structures (Alber, 1992). The second subgrouping is based on domain homology to the intercellular signalling domains of the *Drosophila* Toll and mammalian interleukin-1 receptors (reviewed by Dangl and Jones, 2001). These kinases show dual recognition. For example the *RPM1* gene product, from *Arabidopsis*, can recognize the two non-homologous Avr genes from *Pseudomonas syringae* (Bisgrove *et al.* 1994). Dual recognition has also been observed with the tomato gene *Mi*, which confers not only nematode resistance but also aphid resistance (Rossi *et al.* 1998).

Another class of *R* gene products is the transmembrane receptor kinase, where both a LRR domain and a protein kinase domain are found on the same protein. The rice Xa21 (Song *et al.* 1995) and the *Arabidopsis* FLS2 gene (Gomez-Gomez and Boller, 2000) are the only members of this class at the

present time. Xa21 and FLS2 encode an apparent LRR-receptor kinase, in which the N terminal LRRs are arranged extracellularly. This region is thought to be joined to a cytoplasmically located protein kinase domain by a transmembrane region (reviewed by Bent, 1996).

The final class of *R* genes is the serine-threonine kinases. The first to be characterized, the *Pto* gene in tomato, encodes a serine/threonine kinase which confers resistance to *Pseudomonas syringae* strains containing *avrPto* (reviewed by Dangl and Jones, 2001). This protein also contains a putative myristolation site, which could provide a membrane anchor (Martin *et al.* 1993). When the *Pto* protein was used in a yeast two-hybrid experiment, *Pti1*, a second serine-threonine protein kinase was identified (Zhou *et al.* 1995). This protein kinase also participates in the *avrPto*-specific defense response and was identified as a substrate for phosphorylation by *Pto* and autophosphorylation. This data suggests that *Pti1* acts downstream of *Pto* in a protein kinase cascade.

The pathogen counterparts to the *R* genes have also been studied. The first pathogen *Avr* gene was cloned more than 10 years ago from maize (Meeley *et al*, 1992), and since then many more have been identified. It now appears that *Avr* gene products can directly serve as defense-eliciting ligands. Particular research efforts have been concentrated on two model systems: infection of tobacco with tobacco mosaic virus (TMV)(Taraporewala and Culver, 1996) and infection of tomato plants expressing the *Cf-9* gene by *Cladosporium fulvum* carrying the *Avr9* gene (van Kan *et al*, 1991). The nature of the ligand can range from oligosaccharides to glycoproteins to fatty acids (reviewed by Ebel and

Mithofer, 1998). In the tobacco host system, the R gene is called the *N* gene and is required for the recognition of the TMV replicase protein (the *Avr* gene product) by the host plant. The *N* gene product is located in the cytoplasm, which makes it suitably placed for the recognition of the TMV-derived *Avr* protein (Parker and Coleman, 1997). Plants, which are lacking this R gene, fail to produce an HR and display a systemic spread of the virus (Lam *et al.* 2001). The *Avr9* protein elicitor is actually a 28-amino acid peptide formed from a larger *Avr9* translation product by proteolytic cleavage (van Kan *et al.* 1991). However both resistant tomato lines and lines without the *Cf-9* gene express a high-affinity binding site for the *Avr9* elicitor (Kooman-Gersmann, *et al.* 1996). Thus direct and unique correlations between *avr9* and *Cf-9* proteins are difficult to draw at this time.

1.6.2 Detection methods of kinase activity and phosphorylation levels

Phosphorylation is an effective procedure for protein modification at a post-translation level. This phosphorylation is conducted by protein kinases thus, playing a crucial role in various plant metabolisms, and more specifically plant defense. Characterization of this role has become essential to the better understanding of the plant defense response. Various methods are currently being used to observe specific kinase activity, yet those only pertinent to this thesis are discussed below.

Firstly, specific antibodies have been frequently used for the characterization of proteins following western detection experiments (Seo *et al.*

1999). The results found are of a quantitative nature, thus indicating whether the treatment causes a variation in the level of the protein of interest. Antibodies range from highly specific, i.e., react only with MAP p42 kinase, to generalist, i.e. reacting with most phosphorylated threonine residues, thus discerning between phosphorylated and non-phosphorylated residues.

Secondly, the activity of the protein kinase of interest is also an important piece of information. In-gel kinase assays were first developed by Geahlen *et al* (1986), where detection of kinase activity on SDS gels was possible after renaturation of the enzyme. The use of crude protein extracts on denaturing gels allows for the addition of artificial protein substrates in cases where substrate specificity of the kinase has been well characterized. And the use of radiolabelled ATP allows the method to measure either phosphorylation of the artificial substrate or autophosphorylation. The results are of a qualitative nature, where one sees either higher or lower kinase activity in the sample.

Finally, 'in-tube' kinase assays were developed by incubating either crude protein extracts or isolated proteins, with a reaction buffer, radiolabelled ATP and an artificial substrate. Kinase mediated phosphorylation of an artificial or a natural substrate found in the extract is measured, e.g., Silva *et al* (2001) measured the activity of purified BNK1 from *Brassica napus* using this technique.

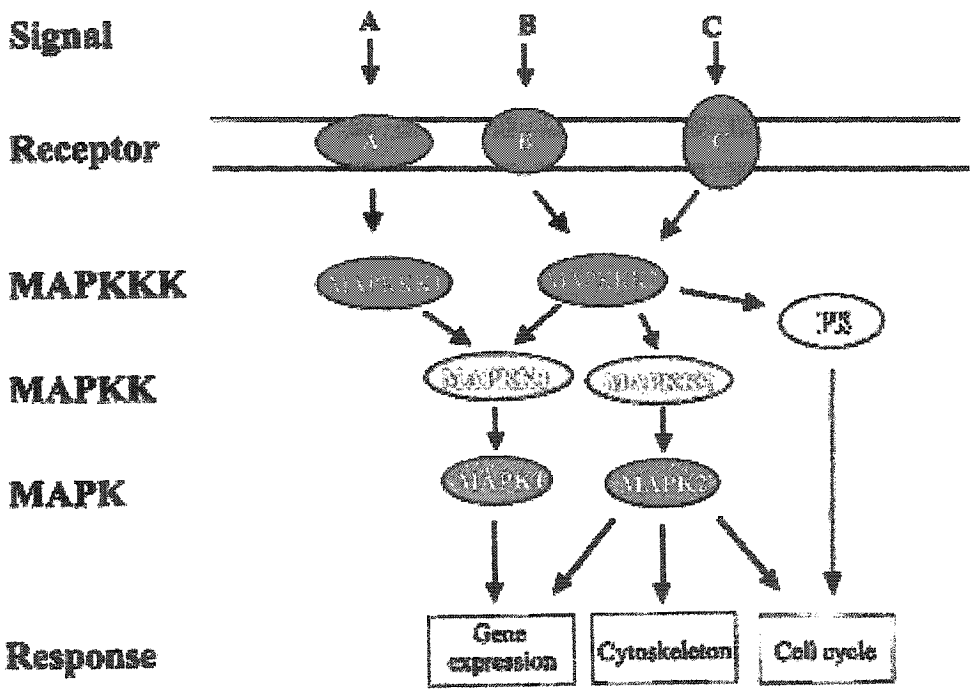
1.6.3 The involvement of MAPKs in plant defense signalling

The involvement of mitogen-activated protein kinases (MAPKs) in a variety of cellular processes in both mammalian and non-mammalian

environments is well documented. This has led to plant defense research involving MAPKs. MAPKs are part of a large family of serine/threonine protein kinases found in all eukaryotes. All serine/threonine kinases present eleven conserved regions, known as kinase domains (Hanks *et al.* 1988). MAPKs are activated by dual-specific MAPK kinases (MAPKKs) through the phosphorylation of conserved threonine and tyrosine residues, within the TXY motif located near kinase domain VIII (reviewed by Jonak *et al.* 1994). Phosphorylation of both residues is required for full activation of the MAPKs. Inactivation of MAPKs occurs through the dephosphorylation of both residues either by serine/threonine phosphatases, tyrosine phosphatases or dual specificity phosphatases (Canagarajah *et al.* 1997). Protein phosphorylation is Ca^{2+} dependent (Ligterink *et al.* 1997), as confirmed with the help of Ca^{2+} channel blockers (Takezawa, 1999).

MAPKKs are in turn activated by MAPKKKs through phosphorylation of serine and serine/threonine residues in the conserved SXXXS/T motif (Figure 5). A specific set of three functionally interlinked protein kinases (MAPKKK-MAPKK-MAPK) form the basic module of a MAPK pathway (reviewed by Jonak *et al.* 1999). MAPK pathways may also be integrated with a variety of upstream signals through interactions with other kinases or G proteins that help to couple the extracellular stimuli detected by plasma membrane receptors and cytoplasmic MAPK modules (reviewed by Hirt, 2000). Downstream of the MAPK cascade, the pathway controls cellular responses. MAPK can either translocate

Figure 5 : General MAPK cascade following various types of stimuli. Taken from Hirt *et al*, 2000.


Signal
A
B
C
Receptor
MAPKKK
MAPKK
MAPK
Response
Gene expression
Cytoskeleton
Cell cycle

into the nucleus and phosphorylate, thereby activating specific transcription factors or remain in the cytoplasm and phosphorylate other enzymes such as protein kinases, phosphatases or cytoskeletal components (reviewed by Ligterink, 2000). Therefore, the MAPK cascades mediate signalling following extracellular stimulation, and can elicit a large variety of specific responses (reviewed by Hirt, 2000).

Different MAPK pathways exist for different types of signals. A model organism for understanding the MAPK pathways has been yeast. Of the six MAPK genes found in the yeast genome, functions for five of them have been identified. For example FUS3 and KSS1 MAPKs are activated by exposure of yeast to pheromones. Yeast carrying mutations in these pathway components are found to be sterile. MPK1 is a MAPK, which is required for the adaptation to hypo-osmolar conditions, while HOG1 is required under hyper-osmolar conditions. Finally, SMK1 is a MAPK, which is involved in spore formation. From studies in yeast, it is now known that different MAPKs cannot substitute for each other and that specific interactions with other proteins are made possible by the action of scaffold proteins (Ruis and Schuller, 1995).

Plant MAPKs can be subdivided into 5 groups (PERK1-5), where unique residues define each subgroup. There is evidence to suggest that the sequence similarities within each subgroup are related to substrate specificity and function (reviewed by Jonak *et al.* 1999). Yet some characteristics are common to all plant MAPKs. All plant MAPKs contain 11 kinase domains and the threonine and tyrosine residues essential for MAPK activation (reviewed by Hirt, 2000).

1.6.3.1 MAPK activation following abiotic stresses

Plants are exposed to numerous types of abiotic stresses such as changes in temperature, water conditions, radiation and wind. Experiments with *Arabidopsis* leaves showed that mechanical manipulation such as touch, induced the transcription of AtMPK3 (a MAPK), MEK1 (a MAPKK) (Morris *et al.* 1997), AtMPK1 (a MAPKKK) as well as an S6 ribosomal protein kinase gene, suggesting a role for the MAPK pathway in the response to mechanical stimuli (Mizoguchi *et al.* 1996; Bogre *et al.* 1996). In alfalfa, it was shown that cold and drought stresses activated a MAPK homologue, MMK4. MMK4 was transiently activated at the level of transcription but no change in MMK4 steady state protein activity levels were observed, thus indicating a role for post-translational regulation (Jonak *et al.* 1996). In tobacco cells, a MAPK was identified as being activated by osmotic stress. Salicylic acid-induced protein kinase (SIPK), a MAPK-homologue, was activated by both hypo-osmotic and hyper-osmotic stress, indicating that the same kinase may play a role in both signalling pathways (Droillard *et al.* 2000; Mikolajczyk *et al.* 2000). SIPK was also found to be transiently and rapidly activated by cutting (Usami *et al.* 1995). High salinity caused the activation of SIMK (salt-induced MAPK) in alfalfa (Kiegerl *et al.* 2000). An upstream regulator of SIMK, SIMKK, was identified using the yeast-two hybrid system (Kiegerl *et al.* 2000). Finally, extensive yeast-two hybrid analysis has allowed for the identification of the first Arabidopsis MAPK cascade, consisting of AtMEKK1, AtMEK1/AtMKK2 and AtMPK4. This was proposed following

experiments showing that AtMPK4 was activated by AtMEK1 *in vitro* and *in vivo* (Huang *et al.* 2000; Mizoguchi *et al.* 1998). Later experiments showed that AtMPK4 and AtMPK6 were activated by low temperature, low humidity, hyper-osmolarity, touch and wounding in *Arabidopsis* (Ichimura *et al.* 2000).

Finally, the involvement of a MAPK in maize was confirmed when Berberich *et al.* (1999) showed that ZmMPK5 was activated during senescence and during temperature regulation after a low-temperature stress.

Thus abiotic stresses cause the activation of distinct MAPK pathways in various organisms.

1.6.3.2 MAPK activation following biotic stresses

Plants must respond to numerous biotic stresses such as wounding and elicitor molecules produced following pathogen infection. For example, a tobacco gene encoding a wound-induced mitogen-activated protein kinase (WIPK) was transcriptionally activated in response to wounding. In transgenic tobacco where the *wipk* gene was suppressed, the accumulation of jasmonic acid or its methyl ester was also suppressed (Seo *et al.* 1999). This result indicates that activation of WIPK is required for triggering the jasmonic-based signal transduction pathway that occurs when tobacco plants are wounded. It was also found that WIPK was activated by infection with TMV (Seo *et al.* 1999). Yet this activation was dependent on the disease resistance gene *N* since infection in tobacco lacking the *N* gene failed to activate WIPK (Zhang and Klessig, 1998). The fact that WIPK is activated by *N* gene-mediated response

suggests that this MAPK is an important component of the pathway leading to TMV resistance. In tobacco it was demonstrated that cryptogein, a *Phytophtora cryptogea* elicitor, caused the induction of SIPK (salicylic acid-induced protein kinase (Zhang and Klessig, 1997). The same SIPK was sufficient to activate defense-related genes and produce a hypersensitive response-like cell death. The SIPK activity was dependent on its phosphorylation by the upstream kinase, SIPKK (Lui *et al.* 2000; Zhang and Liu, 2001). In recent studies, both MAPKs, SIPK and WIPK were shown to be activated by a MAPKK, NtMEK2 suggesting that activiation occurs via a phosphorylation cascade. This putative cascade was shown to be activated by cryptogein response and pathogen defense response (Yang *et al.* 2001). In alfalfa cells, four MAPKs, SIMK, MMK2, MMK3 and SAMK, were activated following the addition of yeast cell wall derived elicitors containing chitin, ß-glucan, ergosterol and N-acetylglucosamine. Chitin mainly activated SIMK, MMK2 and MMK3 and ergosterol mainly activated SIMK, MMK3 and SAMK while ß-1,3 glucan induced all four MAPKs (Cardinale *et al.* 2000). This differential activation implies that there are specific MAPKs for specific elicitor molecules and thus different pathways.

Using the purified *Cladosporium fulvum* gene product, researchers were able to observe the activation of two MAPKs, WIPK and SIPK, in tobacco cell cultures (Romeis *et al.* 1999). Activation was dependent upon the presence of the defense related gene product *Cf-9*. The accumulation of a WIPK transcript in response to *Avr9* in tobacco plants containing the *Cf-9* gene could also be demonstrated.

The *Arabidopsis* MAPK6 (AtMPK6) is activated by elicitor molecules from bacteria, fungi and other plants. Four different elicitor molecules produce a rapid and transient activation of this kinase.

Finally, researchers have identified a MAPK-homologue in wheat, WCK-1, which is induced by elicitor molecules from *Typhula ishikariensis*, suggesting a possible role in pathogen-related response in wheat (Takezawa, 1999).

In a strange turn of events the roles of MAPKs have broadened. Recently published work has shown that *Arabidopsis mpk4* mutants (generated by the insertion of transposable elements) exhibit the properties of constitutive systemic acquired resistance (SAR) including elevated salicylic acid levels, increased resistance to infectious pathogens and constitutive expression of pathogenesis-related genes. Thus MPK4 kinase activity may be required to suppress SAR (Petersen *et al.* 2000). This negative regulation has also been identified with the Arabidopsis gene *enhance disease resistance 1* (*edr1*). Mutations in this gene confer resistance to powdery mildew caused by *Erysiphe cichoracearum.* Resistance mediated by the *edr1* is correlated with the induction of several defense responses, including host cell death. When the EDR1 gene was isolated, it was found to encode a putative MAPKKK similar to CTR1, a negative regulator in the ethylene response. Orthologues of EDR1 are found in monocots, e.g., rice, barley, indicating the possibility of similar, negative regulation in a wide range of crops (Frye *et al.* 2001).

1.6.4 The involvement of CDPKs in plant defense signalling

Calcium (Ca^{2+}) is a ubiquitous intracellular messenger in plant cells which responds to a variety of stresses such as wind, touch, gravity, cold, auxin, light, abscisic acid, salt stress and fungal elicitors (reviewed by Hardie, 1999). Four types of protein kinases constitute the CDPK superfamily: Ca^{2+}-dependent protein kinases (CDPKs), calmodulin-dependent protein kinases (CaMKs), kinases with a combination of both activities (CCaMKs) or neither (CRKs or Ca^{2+}-related protein kinases) (reviewed by Harmon *et al.* 2000). CDPKs contain three functional domains: catalytic, autoinhibitory and calcium binding (Harmon *et al.* 1994). The calcium-binding domain of archetypal CDPK shows strong similarity to calmodulin, and contains four EF-hands calcium-binding motifs (reviewed by Harmon *et al.* 2000). Plant CDPKs have a junction region between the kinase catalytic domain and the calmodulin-like domain (CLD), in a similar position to the autoinhibitory region found in the mammalian CaMKs. There is now evidence that this junction region serves as an autoinhibitory sequence whose effects are relieved by the binding of Ca^{2+} to the CLD of the same polypeptide (Harmon *et al.* 1994). Yet some plant CDPKs do lack the CLD (reviewed by Hardie, 1999).

Intramolecular binding of Ca^{2+} to the CLD disrupts the structure of the autoinhibitory sequence causing a release of inhibition (activation) of CDPK (Huang *et al.* 1996). Both native and recombinant CDPKs exhibit intramolecular autophosphorylation activity. Although many CDPKs contain potential phosphorylation sites (Lys-Gln-Phe-Ser) within the autoinhibitory domains,

activation of CDPKs via phosphorylation has not been directly demonstrated. Autophosphorylation of CaMKs at sites within the autoinhibitory domains similar to those in CDPKs has yielded active enzymes, which no longer require calmodulin (reviewed by Harmon *et al.* 2000). Autophosphorylation of groundnut CDPK is required for its activity, yet it occurs at low Ca^{2+} concentrations and might not have a regulatory role *in vivo* (Chaudhuri *et al.* 1999). In contrast, the autophosphorylation mechanisms of the CDPK are inhibitory in the winged bean (*Psophocarpus tetragonolobus*). Therefore, clear and consistent evidence showing a role for autophosphorylation in CDPK activation has not yet emerged.

The first Ca^{2+}-dependent protein kinase (CDPK) isolated from soybean was inducible by Ca^{2+} but independent of calmodulin (Harmon *et al.* 1987). Additional examples of CDPKs are presented below. Efforts were then concentrated on the roles of CDPKs in plant response to the environment. In rice, a gene encoding a CDPK (OsCDPK7) was identified as being activated by cold and salt stresses. The cold and salt/drought tolerances were well correlated with the level of OsCDPK7 protein expression. Over-expression of OsCDPK7 in transgenic rice lead to an enhanced induction of stress-responsive genes following salinity/drought but not to cold stress suggesting that the downstream pathways leading to salt/drought tolerance and cold tolerance are independent of each other (Saijo *et al.* 2000). The expression of the tomato full-length cDNA, LeCDPK1, was enhanced in detached leaves treated with pathogen elicitors and hydrogen peroxide (H_2O_2). Moreover, mechanical wounding of tomato plants caused a transient increase in LeCDPK1 mRNA that correlated with an increase

in the amount of kinase activity detected (Chico *et al.* 2002). In tobacco, it was found that exposure to non-specific elicitors (chitin fragments) and mechanical wounding caused an increase in transcript levels of NtCDPK1 (Yoon *et al.* 1999). In transgenic tobacco, the *Cf-9/Avr9* race specific elicitor response caused an increase in NtCDPK1 transcript levels. This CDPK showed a rapid transient increase and interconversion to an active form after pathogen elicitation and hypo-osmotic stress. Also, the function of the CDPK was further investigated following virus-induced gene silencing (VIGS). In CDPK-silenced plants, the response to the addition of race-specific elicitor was reduced. Silencing resulted in a loss in NtCDPK2 mRNA (Romeis *et al.* 2001) and delayed the hypersensitive response. A tobacco 68/70 kDa CDPK was also found to be induced by the *Cf-9/Avr9* race specific interaction. This membrane-bound CDPK was induced 5 min. after the addition of the elicitor, during in vivo stimulation (Romeis *et al.* 2000).

A similar elicitor-dependent response was observed in French bean cells cultured *in vitro*. After treatment with an elicitor preparation from the fungus *Colletotrichum lindemuthianum*, a 55-kDa CDPK was induced (Allwood *et al.* 1999). Finally, transcript level of a maize CDPK, ZmCPK10, was found to be induced by infection with *Fusarium moniliforme* or treatment with fungal elicitors from it (Murillo *et al.* 2001). Activation of this kinase leads to increased levels of *PRms* mRNA, where *PRms* is a pathogenesis-related protein. Thus, the involvement of ZmCPK10 in signal transduction in the defense-related pathway has been proposed.

While evidence for the involvement of CDPK in stress response in plants is unequivocal, the amount of published literature is relatively small, making the comparison between different host systems difficult (see Figure 6 for summary of functions).

1.6.5 The involvement of other types of kinases in plant defense signalling

A number of other types of kinases have been implicated in plant defense signalling. In *Arabidopsis*, a gene coding for a receptor-type kinase was isolated and identified as a pathogenesis-related protein. The PR5K gene codes for a predicted kinase containing a transmembrane domain and an intracellular serine/threonine kinase domain. The extracellular domain is related to the PR5 proteins, and the PR5K transcripts accumulate at low levels in all tissues except roots (Wang *et al.* 1996).

Protein kinase C (PKC) is a member of a family of kinases, which has been implicated in plant defense. Family members contain four conserved domains (C1-C4) with the following possible functions: C1 contains a cystein-rich motif, which forms a diacyl glycerol/phorbol ester-binding site. C2 contains the recognition sites for acidic lipids and the Ca^{2+}-binding site. C3 and C4 domains contain the ATP-binding site and the substrate-binding site of the kinase core, respectively (Newton, 1995). Subramaniam *et al* (1997) found in potato tuber discs, that the activation of pathogenesis-related protein PR10a was positively controlled by a PKC. This kinase affected the binding of nuclear factors PBF-1 and PBF-2 (Pr-10a binding factors) to a DNA-specific elicitor response element

(ERE). Using inhibitors of PKC, they were able to abolish the elicitor-induced binding of the PBF-2 to the ERE. This correlated with a decrease in PR-10a protein accumulation. In maize, a protein kinase C was also identified (ZmcPKC70) (Chandok and Sopory, 1998). It was found that this kinase was induced by the presence of oleyl acetyl glycerol (OAG) in the presence of Ca^{2+} and was inhibited by phorbol 12-myristate 13-acetate (PMA).

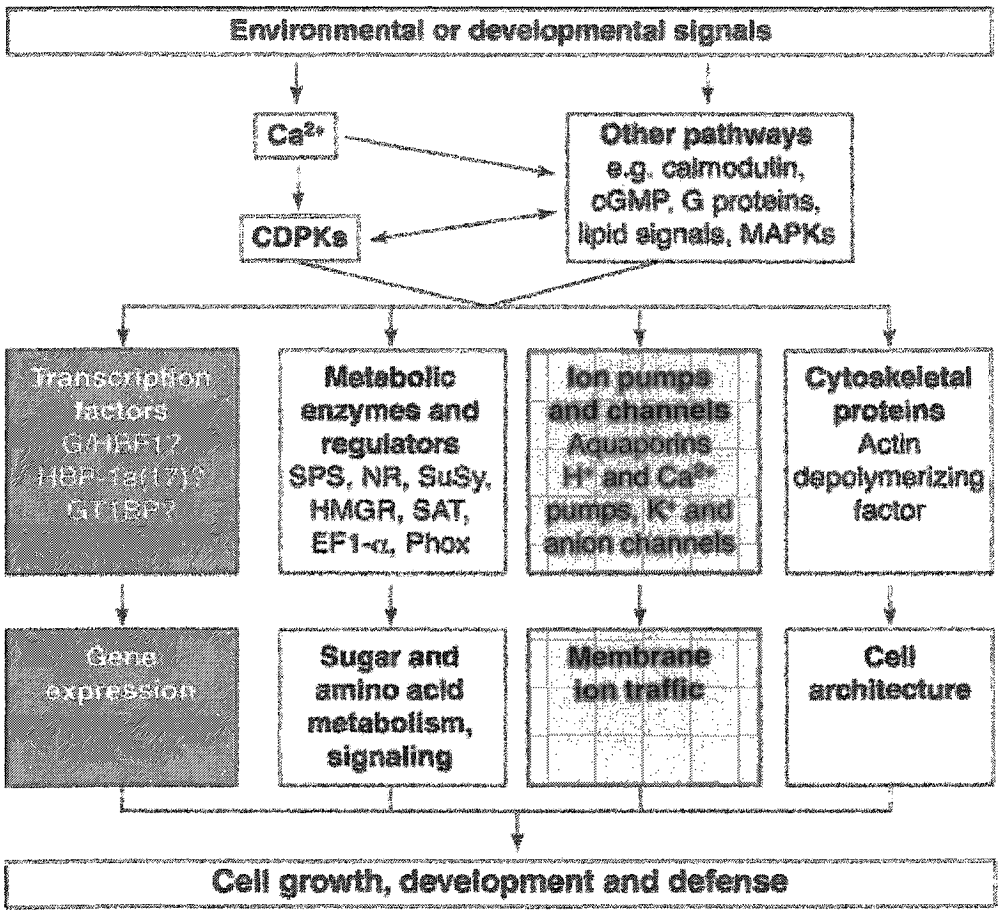
A glycogen synthase kinase (GSK) was found to be involved in plant defense, more specifically in alfalfa leaves. Wounding activated GSK-3; a wound induced GSK (WIG) that encodes a functional GSK-3 gene (Jonak *et al.* 2000).

A novel soybean cDNA has been isolated encoding a bZIP protein, G/HBF-1, which binds to the G-box and the adjacent H-box regions of the chalcone synthase *chs15* promoter. G/HBF-1 is quickly phosphorylated in elicited soybean cells, and almost exclusively on serine residues. It was proposed that the activation of G/HBF-1 kinase via its phosphorylation is a terminal events in a pathway for activation of early plant defense response (Droge-Laser *et al.* 1997).

Finally, the role(s) of a novel group of *Arabidopsis* proteins in pathogen- and salicylic acid-induced responses, in *Arabidopsis* have been observed. These WRKY proteins have downstream targets identified as receptor-like kinase (RLKs) genes. All four of these RLKs were activated by salicylic acid and by bacterial infection.

The WRKY proteins recognize the W-box sequence [(T)TGAC(C/T)] of the

Figure 6: Summary of possible functions of CDPKs in a plant environment. These functions depend on the type of stimulus as well as the intended purpose. Taken from (Harmon *et al*, 2000).



Trends in Plant Science

RLKs within a few hundred base pairs upstream of the coding sequences. Thus, the W-box binding proteins that are induced by salicylic acid or by pathogen infection may regulate genes encoding defense proteins (Du and Chen, 2000).

Table 1 provides a summary of the kinases previously discussed, as well as their putative role(s) in plant defense. In closing, the involvement of different types of kinases in plant defense is well documented. Information concerning the role each play is crucial to the development of a solid understanding of signalling pathways involved in stress response.

1.7 The objectives of this study

The short-term objective of this study was to look at the involvement of protein kinases in wheat defense mechanisms after point- and spray inoculations with a fungal pathogen, such as *Fusarium graminearum*. This was conducted by analysing the protein phosphorylation patterns with antibodies specific for phosphorylated tyrosine, threonine and serine residues and by analysing protein kinase activity via in-tube and in-gel kinase assays. An attempt to clone a specific kinase and look at RNA levels also indicated a possible post-transcriptional activation.

The long-term objectives of this study were to determine whether differences in defense mechanisms against fungal infection exist between

Table 1: Summary of known kinases interacting in plant/pathogen interactions with their putative targets.

Protein kinase	Type of kinase	Plant/Pathogens	Putative targets	References
FLS2	*LRR-kinase*	*Arabidopsis thaliana* / bacterial flagellin	unknown	Gomez-Gomez and Boller, 2000
Xa21	*LRR-NBS kinase*	rice/*Xanthomonas campestris*	unknown	Song *et al*, 1995
Lr10	*Receptor-like kinase*	wheat/*Leaf rust*	unknown	Feuillet *et al*, 1997
RLK4	*Receptor-like kinase*	A. thaliana / Pseudomonas syringae	unknown	Du *et al*, 2000
G/HBF-1	Serine kinase	soybean / *P.syringae*	*chs15* promoter	Droge-Laser *et al*, 1997
Pto	*Ser/thr kinase*	*tomato* / P.syringae	*Pti* complex	Martin *et al*, 1993
EDR1	MAPKKK negative *regulator*	*A. thaliana / Erysiphe cichoracearum*	unknown	Frye *et al*, 2001
NtMEK2	*MAPKK*	tobacco / fungal elicitor cryptogein	unknown	Yang *et al*, 2001
SIMK and SAMK	MAPK	alfalfa / yeast cell-wall derived elicitors	unknown	Cardinale *et al*, 2000
ERMK	*MAPK*	parsley cells / *Phytophthora sojae*	*PDF1.2 / THI2.1* genes	Ligterink *et al*, 1997
MAPK4	*MAPK negative regulator*	*A. thaliana / P. syringae*, salicylic acid	activates *SIPK* and *WIPK*	Petersen *et al*, 2000
SIPK	*MAPK*	tobacco / *Phytophthora spp*	unknown	Zhang and Klessig, 1997
WCK-1	MAPK	wheat / *Typhula ishikariensis*	unknown	Takezawa, 1999
NtCDPK3	CDPK	tobacco / fungal elicitor Avr-9	unknown	Romeis *et al*, 2001
NtCDPK2	CDPK	tobacco / fungal elicitor Avr-9	unknown	Romeis *et al*, 2001

susceptible and resistant wheat cultivars and to document these differences. The ultimate goal would be to understand the defense mechanisms involved in wheat defense after fungal infection. Such breakthroughs could potentially lead to the development of more resistant varieties for agricultural purposes.

The effects of *Fusarium graminearum* on different cereals have been well documented. The effects of this pathogen on different wheat cultivars will be dependent on the level of susceptibility of the cultivars to infection. We expect that the defense response at the molecular level will also differ with cultivar susceptibility. In this study, the use of two different wheat cultivars, Frontana (resistant) and Roblin (susceptible) will produce comparative results. Following spray inoculations of wheat heads within a time-course experiment, we hypothesized that there would be differences in phosphorylation levels and/or phosphorylation activity between the two cultivars and between the mock and fungal inoculated samples. These differences could be associated with different defense mechanisms corresponding to susceptibility levels.

In combination with the above approach, molecular cloning of a MAPK homologue was proposed. This permitted us to observe variation in transcript levels following infection in the selected cultivars. As described above, kinase activation occurs as a post-translational event, thus activating proteins already formed. Based on previous observations in the other plant systems as described above, we hypothesize that differences in MAPK-specific transcript levels, between cultivars following infection, will be negligible.

Chapter 2

Materials and Methods

The composition of buffers and solutions is given in Appendix A.

2.1 Plant and fungal materials

Wheat cultivars Frontana and Roblin were grown in a chamber with a 16 h: 8 h, light:dark cycle at 25°C. A suspension of fungal spores (~ 50 000 spores/ml from *Fusarium graminearum* strain DAOM 178 348, provided by Dr. R. Pandeya, ECORC) was used for all experiments, both spray and point-inoculations.

Two different wheat cultivars were used for the inoculation experiments: Roblin (susceptible variety) and Frontana (resistant variety). In a typical field inoculation experiment, it was observed that the disease incidence after spray inoculation were 80% in Roblin and 30% in Frontana. The disease incidence indicates the proportion of wheat heads in a plot that show symptoms. The disease severity fro each cultivar was 50% in Roblin and 20% in Frontana; where disease severity indicates the proportion of infected florets on infected heads (personal communication, George Fedak).

2.2. Inoculation with fungal spores

For spray inoculation experiments, 1-2 ml of spore suspension was sprayed onto heads that had reached the mid-anthesis growth stage. Plants grown in the greenhouse were then kept under mist (10 s every 15 min) for the duration of the

experiment in an attempt to reproduce conditions most favorable to disease in nature. Spraying of wheat heads partially mimics the natural infection process, yet the amount of spores sprayed onto heads greatly surpasses the amount found naturally. In control experiments, sterile water was sprayed onto the heads and the plants were kept under similar misting conditions but in a separate greenhouse. Whole heads were harvested (10 heads per time-point) at several time points (0, 1, 6, 12, 24, 48 h and 6 days after inoculation), immediately frozen in liquid nitrogen and stored at -80°C.

For point inoculation experiments, 10 µl of spore suspension was introduced with a micro-pipetter between the inner and outer glume of selected spikelets from heads that had reached the mid-anthesis stage. Only the spikelets on one side of the florets were point-inoculated. As before, plants grown in the greenhouse were then kept under mist for the duration of the experiment. Control experiments consisted of spikelets point-inoculated with 10 µl of sterile water between the inner and outer glumes, and kept under mist conditions in a separate greenhouse. For both treatments, the inoculated spikelets were harvested after 6 and 24 h, immediately frozen in liquid nitrogen and stored at -80°C.

2.3 Plant protein extractions and quantification

The frozen tissue was ground to a fine powder with the aid of a porcelain mortar and pestle and then stored at -80°C until further manipulations. Aliquots of the ground tissue were transferred to a sterile 1.5 ml Eppendorf tube and kept on

dry ice until the addition of protein extraction buffer containing 50 mM Tris-HCl pH 7.0, 2% sodium dodecyl sulfate (SDS) (w/v), 10% glycerol (v/v), 1% ß-mercaptoethanol, 10 mM dithiothreitol (DTT), 1mM phenylmethyl-sulfonyl fluoride (PMSF), 5 µg/ml of antipain, 5 µg/ml of leupeptin and 5 µg/ml of aprotinin (Gleddie, personnal communication). Extraction buffer was added to the ground tissue at the rate of 400 µl per 200 mg of ground tissue and the sample was vortexed for 30 s to ensure adequate mixing. The mixture was then centrifuged at 4°C at 13, 500 rpm for 25 min in a Beckman Coulter microcentrifuge. The supernatant was transferred into a sterile microfuge tube and stored frozen at -80°C until required.

2.4 SDS Gel Electrophoresis

A 15% poly-acrylamide gel was prepared following a standard recipe (Laemmli, 1970) using a Bio-Rad Protean Mini-Cell II SDS-PAGE system. An aliquot of the total protein extract sample (10 µl) was added to an equal volume of protein loading buffer and placed in a block heater (Techne Genius thermocycler) at 99°C for 5 min. The sample was then briefly centrifuged and loaded onto the gel. Following immersion in electrophoresis buffer, the gel was electrophoresed at 100 V for approximately 2 h, or until the dye nearly reached the bottom of the gel. In order to estimate the size of the bands, the 10 kDa protein ladder from Invitrogen was included as a molecular weight marker.

The gel was then removed from the apparatus, transferred into a plastic container containing Coomassie blue G-250 staining solution and shaken overnight

on a Thermolyne Rotomix shaker at 55 rpm. The gel was destained with three changes of 10% acetic acid solution, with shaking.

After destaining, the gel was dried in a Savant SpeedGel SG210D. The gel was placed onto three layers of Whatmann 3MM paper, covered with Saran Wrap and dried under vacuum at 58°C until the gel was completely dry (about 1h 45min).

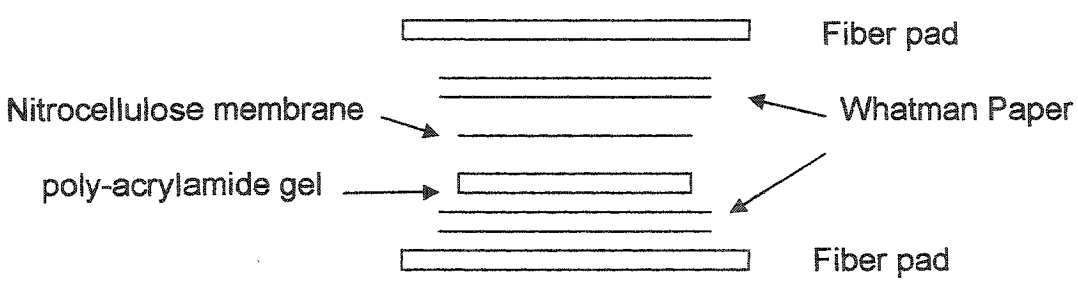
2.5 Western analysis

For western experiments, the same steps were used as far as gel preparation and sample loading as previously mentionned in section 2.4. Commercially available (phosphorylated) proteins were used as positive controls. These consisted of bovine serum albumin (BSA) conjugated with proper phosphorylated residue (Sigma). These were initially diluted from the stock solution into a 1:1000 dilution factor. Then, equal volumes from the diluted phosphorylated protein and protein loading buffer were combined. Molecular markers were used to determine the approximate size of bands, when necessary. This was the Benchmark prestained 10kDa protein ladder from Invitrogen. Each analysis was duplicated with a different protein extract from the same biological sample. In addition, Antoine Leaustic also repeated the experiments on a different biological sample-set of Roblin infected with *Fusarium graminearum* and water (data not shown). The antibodies were specific for phospho-threonine, phospho-tyrosine and phospho-serine residues.

2.5.1 Protein transfer

The transfer apparatus consisted of the Bio-Rad Mini Trans-Blot Cell. Initially, the membrane was pre-equilibrated in transfer buffer. When blotting with the phospho-serine antibody, the Hybond-C extra nitrocellulose membrane (Amersham Pharmacia) was used, while the ECL nitrocellulose membrane (Amersham Pharmacia) was used with the phospho-threonine and phospho-tyrosine antibodies. The fiber pads were initially submerged in transfer buffer, and then two layers of fitted Wathman papers were laid on the fiber pad. The separating gel was then laid on the Whatman papers, ensuring no air bubbles were trapped. The nitrocellulose membrane was then laid onto the separating gel, while still ensuring that no air was trapped between the gel and the membrane. Two layers of Whatman paper were laid onto the nitrocellulose membrane and finally the second fibre pad was added (Figure 6). The "sandwich" was clamped together and inserted in the transfer module. A cooling unit filled with ice, was added to the tank to maintain a low temperature in the transfer buffer. The whole transfer module was submerged in transfer buffer and ran at 100 V for 1.5 h. The nitrocellulose membrane was then removed and placed in a plastic container; with the protein side facing upwards, in blocking buffer at 4°C overnight and covered with Saran Wrap. Blocking buffers consisted of 0.4 g of Gelatine Blocking Buffer (Sigma) (for the phospho-serine antibody) or Casein Blocking Buffer (Sigma) (for the phospho-threonine and -tyrosine antibodies) into 20 ml of 1X TBS + 0.1% Tween 20 (Dr. John Bell, personal communication).

Figure 7: Western transfer setup


Fiber pad
Nitrocellulose membrane
Whatman Paper
poly-acrylamide gel
Fiber pad

2.5.2 Antibodies and detection methods

The following day, the blocking buffer was removed from the container. The excess was then washed with 1X TBS + 0.1% Tween 20 initially for 15 min and then twice more for 5 min each, always changing and adding fresh 1X TBS + 0.1% Tween 20 before every wash. The washes were performed on a Gyrotory Shaker-Model from New Brunswick Scientific at 75 rpm. The primary antibodies were mouse monoclonal phospho-serine, -threonine and -tyrosine antibodies of IgG isotype (Sigma). These antibodies were diluted into 1X TBS + 0.1% Tween 20 at a dilution factor of 1:3000 in a final volume of 10 ml. The solution was then added onto the membrane and the speed of the Gyrotory Shaker-Model from New Brunswick Scientific was increased to 200 rpm, for 2 h. The antibody solution was then removed and replaced with fresh 1X TBS + 0.1% Tween 20, with the shaker rotating at 75 rpm for 15 min. This was followed by two washes of 5 min each with fresh 1X TBS + 0.1% Tween 20 each wash. The secondary antibody consisted of a goat anti-mouse IgG (Bio-Rad), conjugated with the horseradish peroxidase enzyme. The dilution factor for this antibody was 1:3000 in a final volume of 10ml of 1X TBS + 0.1% Tween 20. The secondary antibody solution was added to the membrane and agitated at 200 rpm for 1 h. The membrane was subsequently washed for 15 min with 1X TBS + 0.1% Tween 20 followed by 4 washes of 5 min each with 1X TBS + 0.1% Tween 20. The detection solution was prepared by adding equal volumes of solution A and solution B from the ECL Western Blotting Detection Kit (Amersham Pharmacia). Four ml of detection solution was poured

onto the membrane surface for a duration of 1 min. The membrane was removed from the plastic container and blotted briefly onto Whatmann paper to remove excess detection solution. The membrane was then wrapped in Saran Wrap and positioned in a Biomax film cassette from Kodak. The membrane was exposed to Kodak Biomax film for an amount of time appropriate for proper observation on the film (5 s to 1 min). The membrane was then removed from the cassette and placed into a sealed plastic bag and stored at -20°C.

2.6 In-tube kinase assays

In-tube kinase assays were performed using artificial substrates to determine kinase activity (Silva, *et al*, 2001). A typical 12 µl reaction mixture contained 20mM Pipes pH 7.0, 10 mM magnesium chloride ($MgCl_2$), 2 mM manganese chloride ($MnCl_2$),100 mg/ml of aprotinin (Sigma), 0.5 mg/ml of artificial substrate, 10 µCi of γ-^{32}P-ATP (3000 mCi/mM)(Amersham) and 6 µl of protein extract. The kinase reaction assay was incubated at room temperature for 30 min after which 6 µl of 3X SDS sample buffer was added to stop the reaction. The samples were boiled for 5 min and then loaded onto a 15% polyacrylamide gel. The gel was run at 100V for approximately 2 h, or until the loading dye had nearly reached the bottom of the gel. The gels were stained, destained and dried as described Section 2.4. The dried gel was exposed to Biomax film at -80°C for a period of time which allowed for an optimal signal to noise ratio (5 min to 30 min). The different artificial substrates used were myelin basic protein (MBP), from bovine brain (Invitrogen)at a

concentration of 0.25 mg/ml, histone III-S from calf thymus (Sigma) at a concentration of 1 mg/ml and casein from bovine milk (Sigma) at a concentration of 1 mg/ml. The positive control consisted of 0.5 µl of active p42 MAPK (erk2)(New England Biolabs) instead of the protein extract. This kinase was naturally found to compete with transport factors for binding to nucleoporins, which mediate the entry and exit of transport factors. It also seems that this kinase enters the nucleus through a direct interaction with nuclear pore complex proteins (Whitehurst *et al*, 2002).

2.7 In-gel kinase assays

The artificial substrate was added to the separating gel solution, prior to casting, which resulted in the substrate being embedded within the 10% poly-acrylamide gel (Hutchcroft, *et al*, 1991). Following electrophoresis of 10 µl samples (Section 2.4) the separating gel was incubated in Buffer A (25 mM Tris-HCl pH 7.5, 0.5 mM DTT, 0.1 mM sodium vanadate (Na_3VO_4), 5mM sodium fluoride (NaF), 0.5 mg/ml bovine serum albumine (BSA) and 0.1% of Triton X-100) for three washes, 30 min each, at room temperature to remove all excess SDS. The kinases were allowed to renature in buffer B (25 mM Tris-HCl pH 7.5, 1 mM DTT, 0.1 mM Na_3VO_4 and 5 mM NaF) overnight at 4°C with three changes of buffer during this period. The gel was then incubated in kinase activity buffer (25 mM Tris-HCl pH 7.5, 2 mM EDTA, 12 mM $MgCl_2$, 1 mM DTT, 0.1mM Na_3VO_4, 200 nM ATP, 50 µCi of γ-^{32}P-ATP (3000mCi/mM, Amersham) for 60 min at room temperature. The reaction was

stopped and the unincorporated substrate was removed by washing the gel in 5% trichloroacetic acid (TCA) + 1% sodium pyrophosphate ($NaPP_i$) for 6 h with 5 buffer changes. The gel was then dried on a Savant SpeedGel SG210D dryer and exposed at -80°C to Kodak Biomax film for a period of time, which allowed for an optimal signal to noise ratio (5 min to 30 min).

Three artificial substrates were individually tested: MBP, histone III-S and casein.

2.8 RNA and DNA methods

2.8.1 RNA Isolation

Tissue collected from each cultivar at several time points was ground in liquid nitrogen using a sterile mortar and pestle. In a typical extraction, TRIZOL reagent (Invitrogen) was added to the ground tissue (1 mL per 100 mg tissue) in a 1.5 ml microfuge tube. The tube was vortexed for 30 s, incubated at room temperature for at least 5 min and then kept on ice for 10 min. All centrifugations were done at 4°C at 12 000 rpm. Following centrifugation for 10 min, the supernatant was transferred to a sterile 1.5 ml microfuge tube. Chloroform was added to the supernatant (0.2 mL per 1 ml of TRIZOL), the mixture was vortexed for 15 sec and then incubated at room temperature for 2-3 min. The resulting mixture was centrifuged for 15 min. After transfer of the aqueous phase to a sterile 1.5 ml microfuge tube, 0.25 ml of isopropanol and 0.25 ml of 2 M sodium chloride (prepared in diethyl pyrocarbonate [DEPC]-treated water) was added to the

supernatant. The solution was mixed by inversion, incubated at room temperature for 10 min and spun for 10 min. The supernatant was then removed and the pellet washed with 75% ethanol (made with DEPC-treated water). One ml of ethanol was added for every ml of TRIZOL initially used. After vortexing, the tubes were spun for 2 min. The supernatant was removed, the pellet was briefly vacuum-dried and resuspended in DEPC-treated water (100 µl for 100 to 500 mg of tissue). Incubation at 60°C and vortexing were employed to dissolve the pellet.

The quality and the recovery of RNA were estimated by spectrophotometry and gel electrophoresis.The concentration of RNA was calculated using the formula 1 O.D. (at 260nm) = 40 µg/ml. An RNA aliquot was also loaded on a 1.5% agarose gel prepared in TAE buffer to further judge the RNA quality.

2.8.2 DNA Isolation

Tissue was ground with the aid of a sterile mortar and pestle; 1.6 g of powder was transferred to a 50 ml Falcon tube and 30 mL of extraction buffer (Qianfa Chen's Extraction Buffer –QCEB, Dellaporta *et al*, 1983) prewarmed to 65°C was added. After thorough stirring with a sterile glass rod, 2 ml of 20% SDS was added. Following vigorous mixing, the extraction was incubated at 65°C for 10 min. Ten ml of 5M potassium acetate were added, then the mixture was vigorously shaken and the tube was placed on ice for 20 min. The sample was centrifuged at 9000 rpm for 20 min at 4°C in a Beckman JA-12 rotor or equivalent. The supernatant was recovered, filtered through Miracloth (InterSciences) and divided into 2 sterile 50 ml

Falcon tubes. Cold 95% ethanol was added to fill the tubes that were then mixed gently by inversion and incubated at -20°C overnight. The DNA was recovered on a bent Pasteur pipette, transferred to a 15 ml tube containing 70% ethanol and incubated at -20°C. The washed DNA was centrifuged at 2000 rpm for 10 min at room temperature and was transferred to a 1.5 ml microfuge tubes with the aid of a sterile glass rod. The pellet was briefly vacuum-dried and dissolved in 400-1000 µl prewarmed (65°C) Tris-EDTA buffer (TE), pH 8 to achieve a final DNA concentration of 5-10 µg/µl. The quality and recovery of the DNA were estimated by spectrophotometry and gel electrophoresis. The concentration of DNA was calculated using the formula 1 O.D. (at 260 nm) = 50 µg/ml. A DNA sample was also loaded on a 1.5% agarose gel prepared in TAE buffer to further judge the DNA quality.

2.8.3 Primer Design for the Amplification of MAPK by PCR and RT-PCR

The 24-mer forward primer WKN1 (5'-GACGAGGGAGATGGTGGCAAG TAA-3') and the 24-mer reverse primer WKN2 (5'-CGGAGTAGTCGGTG GAGTTGAGCA-3') were designed using seven MAP kinase sequences available in the GenBank database (www.ncbi.nlm.nih.gov). These were *Avena sativa* Asmap1 (Accession # X79993), *Nicotiana tabacum* WIPK (Accession # BAB79636), *Medicago sativa* MMK1 (Accession # Q07176), *Triticum aestivum* MAPK WCK-1 (Accession # AF079318), *Arabidopsis thaliana ATMPK3* MAPK (Accession # Q39023), *Arabidopsis thaliana ATMPK4* (Accession # Q39024) and *Arabidopsis*

thaliana ATMPK6 (Accession # AAB64027). The software DNAMan (Lynnon Biosoft) was used to align all six sequences and produce a consensus sequence from which the primer set was designed. Primers were synthesized by Invitrogen.

Previous work by Berberich *et al*, 1999 had identified eleven domains that were common to the five MAPK amino acid sequences they had aligned. A second set of primers was designed using the sequences found in only domains II and IX common to all MAPKs The primer set consisted of the 24-mer MKOII (5'-GTGGCAAGTAAGAAGATCGCCAAC-3') and the 24-mer MKDIX (5'-GTGATCCTCT GCAGCGGGTTAAA-3') (see figure 8).

PCR and RT-PCR were performed using primers WKN1 and WKN2 followed by the MKO II and MKD IX. The reaction mixture for the PCR contained 1X PCR reaction buffer (Invitrogen), 1 µM of WKN1, 1µM of WKN2, 1.5 mM of magnesium chloride ($MgCl_2$), 0.2 mM of deoxyribonucleoside triphosphate (dNTP) mix (Promega), 100 ng of template DNA and 2.5 units of *Taq* (*Thermus aquaticus*) DNA polymerase (Invitrogen), with the final volume adjusted to 20 µl with deionized water. The mixture was overlayed with mineral oil. The tubes were vortexed, spun briefly, and inserted in a Techne Genius thermocycler. The following program was used:

Pre-incubation:	2 min at 94°C
30 cycles of	
Denaturation:	1 min at 94°C
Annealing:	1 min at 55°C

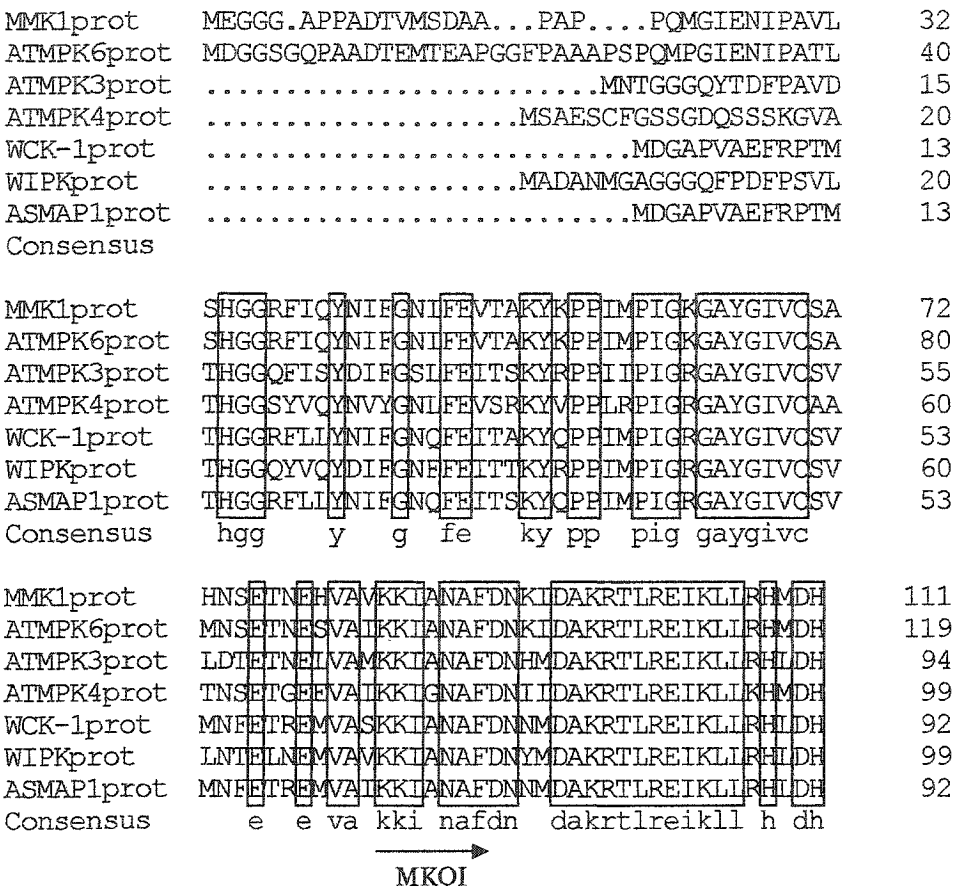
Extension:	2 min at 72°C
Post-incubation:	5 min at 72°C

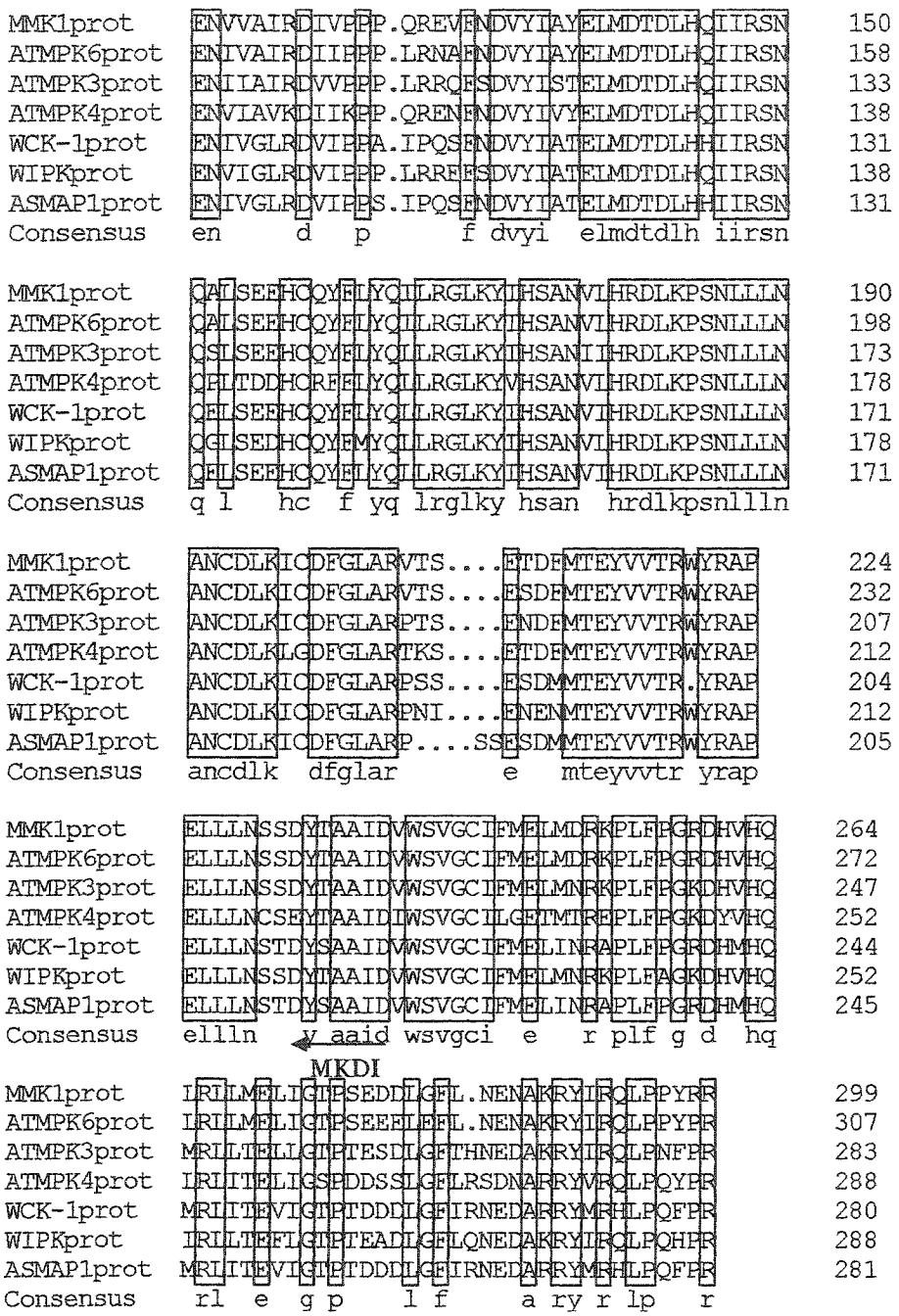
The resulting products were analysed on a 1.5% agarose gel in TAE.

RT-PCR reactions followed the basic protocol provided by Life Technologies for the 5' RACE procedure with some modifications. The RT step was performed as follows: 2.5 pM of both primers were added to 2.5 µg of RNA in a final volume adjusted to 15.5 µl with DEPC-treated water. After incubation at 70°C for 10 min in order to denature the RNA, the mixture was chilled on ice for 1 min and briefly centrifuged. The solution was adjusted to 1X PCR reaction buffer, 2.5 mM $MgCl_2$, 10 mM of DTT and 0.4 mM dNTP mix (all from Invitrogen). After 1 min incubation at 42°C, 1 µl of SUPERSCRIPT II RT (200 units/µL; Life Technologies) was added and the solution was incubated for 50 min at 42°C. The reaction was terminated by heating at 70°C for 15 min and then incubated at 37°C for 30 min with 100 units of RNAase mix (mixture of Rnase H and Rnase T1, Invitrogen) to degrade residual RNA.

cDNA was purified using the GLASSMAX DNA Isolation Spin Cartridge System (Invitrogen). The first strand reaction (above) was mixed with 120 µl of binding solution (6M sodium iodide (NaI)) and transferred to a GLASSMAX spin cartridge. The cartridge was centrifuged at 13,000 rpm for 20 s at room temperature, washed four times with 0.4 ml of cold (4°C) 1X wash buffer (supplied by kit, proprietary, Invitrogen) and twice with cold (4°C) 70% ethanol (all centrifugation steps were for 1 min at 13,000 rpm at room temperature). The

Figure 8: Consensus sequences of 7 MAPKs. *Medicago sativa* MMK1, *Arabidopsis thaliana* ATMPK6, *Arabidopsis thaliana* ATMPK3, *Arabidopsis thaliana* ATMPK4, *Triticum aestivum* WCK-1, *Nicotiana tabacum* WIPK and *Avena sativa* ASMAP1. Consensus sequence and box areas show 100% homology. Arrows designate primer pairs.


MMK1prot MEGGG.APPADTVMSDAA...PAP....PQMGIENIPAVL 32
ATMPK6prot MDGGSGQPAADTEMTEAPGGFPAAAPSPQMPGIENIPATL 40
ATMPK3prot .....MNTGGGQYTDFPAVD 15
ATMPK4prot .....MSAESCFGSSGDQSSSKGVA 20
WCK-1prot .....MDGAPVAEFRPTM 13
WIPKprot .....MADANMGAGGGQFPDFPSVL 20
ASMAP1prot .....MDGAPVAEFRPTM 13
Consensus
MMK1prot 72
ATMPK6prot 80
ATMPK3prot 55
ATMPK4prot 60
WCK-1prot 53
WIPKprot 60
ASMAP1prot 53
Consensus hgg y g fe ky pp pig gaygivc
MMK1prot 111
ATMPK6prot 119
ATMPK3prot 94
ATMPK4prot 99
WCK-1prot 92
WIPKprot 99
ASMAP1prot 92
Consensus e e va kki nafdn dakrtlreikll h dh
MKOI


MMK1prot ENVVAIRDIVPPP.QREVFNDVYIAYELMDTDLHQIIRSN 150
ATMPK6prot ENIVAIRDIIPPP.LRNAFNDVYIAYELMDTDLHQIIRSN 158
ATMPK3prot ENIIAIRDVVPPP.LRRQFSDVYISTELMDTDLHQIIRSN 133
ATMPK4prot ENVIAVKDIIKPP.QRENFNDVYIVYELMDTDLHQIIRSN 138
WCK-1prot ENIVGLRDVIPPA.IPQSFNDVYIATELMDTDLHHIIRSN 131
WIPKprot ENVIGLRDVIPPP.LRREFSDVYIATELMDTDLHQIIRSN 138
ASMAP1prot ENIVGLRDVIPPS.IPQSFNDVYIATELMDTDLHHIIRSN 131
Consensus en d p f dvyi elmdtdlh iirsn
MMK1prot QALSEFHCQYFLYQLLRGLKYIHSANVLHRDLKPSNLLLN 190
ATMPK6prot QALSEFHCQYFLYQLLRGLKYIHSANVIHRDLKPSNLLLN 198
ATMPK3prot QSLSEFHCQYFLYQLLRGLKYIHSANIIHRDLKPSNLLLN 173
ATMPK4prot QFLTDDHCRFFLYQLLRGLKYVHSANVLHRDLKPSNLLLN 178
WCK-1prot QELSEFHCQYFLYQLLRGLKYIHSANVIHRDLKPSNLLLN 171
WIPKprot QGLSEDHCQYFMYQLLRGLKYIHSANVLHRDLKPSNLLLN 178
ASMAP1prot QFLSEFHCQYFLYQLLRGLKYIHSANVIHRDLKPSNLLLN 171
Consensus q l hc f yq lrglky hsan hrdlkpsnllln
MMK1prot ANCDLKICDFGLARVTS....ETDFMTEYVVTRWYRAP 224
ATMPK6prot ANCDLKICDFGLARVTS....ESDFMTEYVVTRWYRAP 232
ATMPK3prot ANCDLKICDFGLARPTS....ENDFMTEYVVTRWYRAP 207
ATMPK4prot ANCDLKLGDFGLARTKS....ETDFMTEYVVTRWYRAP 212
WCK-1prot ANCDLKICDFGLARPSS....ESDMMTEYVVTR.YRAP 204
WIPKprot ANCDLKICDFGLARPNI....ENENMTEYVVTRWYRAP 212
ASMAP1prot ANCDLKICDFGLARP....SSESDMMTEYVVTRWYRAP 205
Consensus ancdlk dfglar e mteyvvtr yrap
MMK1prot ELLLNSSDYTAAIDVWSVGCIFMELMDRKPLFPGRDHVHQ 264
ATMPK6prot ELLLNSSDYTAAIDVWSVGCIFMELMDRKPLFPGRDHVHQ 272
ATMPK3prot ELLLNSSDYTAAIDVWSVGCIFMELMNRKPLFPGKDHVHQ 247
ATMPK4prot ELLLNCSEYTAAIDIWSVGCILGEIMTREPLFPGKDYVHQ 252
WCK-1prot ELLLNSTDYSAAIDVWSVGCIFMELINRAPLFPGRDHMHQ 244
WIPKprot ELLLNSSDYTAAIDVWSVGCIFMELMNRKPLFAGKDHVHQ 252
ASMAP1prot ELLLNSTDYSAAIDVWSVGCIFMELINRAPLFPGRDHMHQ 245
Consensus ellln y aaid wsvgci e r plf g d hq
MKDI
MMK1prot IRILMELIGTPSEDDLGFL.NENAKRYIRQLPPYPR 299
ATMPK6prot IRILMELIGTPSEEELEFL.NENAKRYIRQLPPYPR 307
ATMPK3prot MRLLTELLGTPTESDLGFTHNEDAKRYIRQLPNFPR 283
ATMPK4prot IRLITELIGSPDDSSLGFLRSDNARRYVRQLPQYPR 288
WCK-1prot MRLITEVIGTPTDDDLGFIRNEDARRYMRHLPQFPR 280
WIPKprot IRLLTEFLGTPTEADLGFLQNEDAKRYIRQLPQHPR 288
ASMAP1prot MRLITEVIGTPTDDDLGFIRNEDARRYMRHLPQFPR 281
Consensus rl e g p l f a ry r lp r


MMK1prot QSFQEKFPHVHPEAIDLVEKMLTFDPRKRITVEDALAHPY 339
ATMPK6prot QSITDKFPTVHPIAIDLIEKMLTFDPRRRITVLDALAHPY 347
ATMPK3prot QPLAKLFSHVNPMAIDLVDRMLTFDPNRRITVEQALNHQY 323
ATMPK4prot QNFAARFPNMSAGAVDLLEKMLVFEPSRRITVDEALCHPY 328
WCK-1prot RSFPGQFPKVQPAALDLIERMLTFNPLQRITVEEALEHPY 320
WIPKprot QQLAEVFPHVNPIAIDLVDKMLTLDPTRRITVEEALDHPY 328
ASMAP1prot RPFPGQFPKVQPAALDLIERMLTFNPLQRITVEEALFHPY 321
Consensus f a dl ml p ritv al h y
MMK1prot LTSLHDISDEPVCMTPFSFDFEQH.ALTEEQMKELIYREA 378
ATMPK6prot LNSLHDISDEPECTIPFNFDFENH.ALSEEQMKELIYREA 386
ATMPK3prot LAKLHDPNDEPICQKPFSFEFEQQ.PLDEEQIKEMIYQEA 362
ATMPK4prot LAPLHDINEEPVCVRPFNFDFEQP.TLTEENIKELIYREI 367
WCK-1prot LERLHDVADEPICTDPFSFDFEQH.PLTEDQMKQLIFNEA 359
WIPKprot LAKLHDAGDEPICPVPFSFDFEQQ.GIGEEQIKDMIYQEA 367
ASMAP1prot LERLHDVADEPICTDPFSFDFEQH.PLTEDQMKQLIFNEA 360
Consensus l lhd ep c pf f fe e k i e
MMK1prot LAFNPEYQQ 387
ATMPK6prot LAFNPEYQQ 395
ATMPK3prot IALNPTYG 370
ATMPK4prot VKENPQDSV 376
WCK-1prot LELNPNFRY 368
WIPKprot LSLNPEYA 375
ASMAP1prot LELNPNFRY 369
Consensus np

cartridge was then transferred to a recovery tube and the cDNA was eluted with 50 µl of sterilized, distilled water preheated to 65°C. The column was spun at 13 000 rpm for 20 s room temperature in order to elute the cDNA.

The purified cDNA was used as a template for PCR utilizing WKN1 and WKN2 and MKOII and MKDIX primers. A 25 µl reaction contained 1 X PCR buffer (Invitogen), 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, 0.1 µM of each primer, 1.5 µl of sample cDNA, and 0.5 µl of *Taq* DNA polymerase (5 units/µl). PCR was carried out as described above for PCR using the primers WKN1 and WKN2. The resulting product was analysed on a 1.5% agarose-TAE gel.

2.8.4 Gel Purification of cDNA

DNA fragments were isolated from agarose gels using Costar Spin-X columns. The desired fragment was cut from the gel with the aid of an UV transilluminator and a sharp razor blade. The agarose piece was stored in a clean 1.5 ml microfuge tube and kept at -20°C until needed.

For DNA isolation the agarose was transferred onto a piece of plastic wrap and diced into fine pieces using a razor blade. TE buffer was added to cover the agarose piece (~100-250 µl), and the mixture was placed at -20°C overnight. The agarose mixture was quick-thawed at 37°C for 15 min, and transferred to a Spin-X column with the aid of a spatula. The tube containing the column was centrifuged for 30 min at room temperature in a microfuge, holding the columns horizontally. The flow through liquid was transferred to a fresh tube and the DNA was

precipitated overnight at -20°C with the additon of 0.5 µl of tRNA (10 mg/ml) as a carrier, 0.1 volume of 3M sodium acetate (NaOAc), pH 5.5 and 2.5 volumes of 100% ethanol. The tube was centrifuged for 30 min at 4°C to recover the fragment, which was resuspended in 12 µl of TE buffer. Estimates of DNA recovery were made by electrophoresis of 1-2 µl on a 1.5% agarose gel and comparison with known amounts of reference DNA.

2.8.5 Ligation

The pGEM-T Easy Vector System (Promega) was used for ligation, following manufacturer's protocol. Each 10 µl ligation mixture consisted of 1X Rapid Ligation Buffer (T4 DNA Ligase), 50ng pGEM-T Easy vector, 1 µl of T4 DNA Ligase (3 Weiss units/µl) and PCR-amplified cDNA fragment. The tubes were mixed by pipetting and incubated overnight at 10-12°C.The amount of cDNA fragment used was determined by the following equation:

$$\frac{50 \text{ ng (vector)} \times \text{size of insert}}{3.0 \text{ kb (vector)}} \times \frac{3}{1} = \text{amount of insert in ng}$$

In some cases it was necessary to concentrate the PCR product in order to achieve the calculated amount. Then, the insert was lyophilised for ~ 5 min in a Savant Refrigerated Condensation Trap.

2.8.6 Transformation of DH5α by Electroporation

Ligation mixtures were electroporated into ElectroMAX DH5α-E Cells (Invitrogen) using the BioRad Gene Pulse II System. Prior to electroporation the

competent cells were thawed on ice and 1 µl of the diluted ligation mixture (diluted 1:5 with SOC) was added to 40 µl of competent cells.The mixture was transferred to a chilled cuvette, after which the cuvette was placed into the holder and pulsed. The pulse controller was set to: 25 µF, 200 Ω and 1.7 kV. Immediately following the addition of 1 ml of SOC at room temperature, the cells were transferred into a sterile snap-capped tube (Falcon 14ml) and incubated at 37°C for 1 h with constant shaking. Then 100 µl of the cells were plated on LB-agar plates containing carbenicillin (0.1 mg/ml), 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal)(2 µg/ml) and isopropylthio-β-D-galactoside (IPTG)(2 µg/ml). The remaining cells were concentrated by centrifugation, resuspended in 100 µl of SOC and plated on similar agar plates. The plates were incubated overnight at 37°C. The following day, putative recombinant colonies were selected and grown overnight at 37°C in 5 mL of TB media with constant shaking.

2.8.7 Plasmid isolation

The isolation of recombinant plasmids was done on 2 ml aliquots of overnight cultures using the UltraClean Mini Plasmid Prep Kit from MoBio Laboratories according to the manufacturer's instructions. At the last step of isolation, DNA was eluted with 50 µl of solution 5 (10 mM Tris/NaCl). Isolated plasmid DNA was quantitated and digested with *Eco RI* at 37°C for 1 h to release the insert and run on a 1.5% agarose-TAE gel. Plasmids were sequenced by Canadian Molecular Research Services Inc (Ottawa). The sequences were then compared to MAPK

accessions in the National Centre for Biotechnology Information (www.ncbi.nlm.nih.gov) to ensure similarity with MAPKs.

2.8.8 Radiolabelling of probes

The cloned insert was purified from an agarose gel as described above in Section 2.8.4. For DNA labelling we used the NEBlot Kit from New England Biolabs Inc. and followed their protocol. Template DNA (25 ng) was denatured in a volume of 33 µl by boiling for 5 min followed by chilling on ice for 5 min. The following reagents were added in order: 10X Labelling Buffer was added (supplied in kit, proprietary), 6 µl of dNTP mixture, 50 µCi α^{32}P dCTP (3000 mCi/mM), 1 µl of DNA Polymerase I- Klenow Fragment (5 u/µl). The mixture was incubated for 1 h at 37°C, and terminated by the addition of 20mM EDTA pH. 8.0.

Radiolabelled DNA was purified from unincorporated nucleotides by using a NICK Column from Pharmacia Biotech according to the manufacturer's instructions. The specific activity of the DNA was estimated using a Canberra Packard Liquid Scintillation Counter model. Prior to their use, probes were denatured for 5 min and chilled on ice.

2.8.9 Northern hybridization analysis

RNA samples (10 µg) were run on a 1.2% formaldehyde/agarose gel (1.2% agarose, 10X MOPS, DEPC-treated ddH_2O, 0.66M formaldehyde) at 60V for approximately 3 h and transferred to a nylon membrane via a standard capillary

transfer method (Maniatis *et al*, 1989). The RNA was cross-linked to the membrane by exposing it to 1200 J of ultraviolet light using the Ultraviolet Crosslinker from ULTRA-LÛM.

Prehybridization for 4-16 h at 42°C was carried out in a solution containing 50% deionised formamide, 5X Denhardt's solution, 1X SDS, 6X SSC and 100 µg/ml of heat-denatured salmon sperm DNA. Hybridization continued for 8-12 h at 42°C in a prehybridization solution with the addition of 5X of Denhardt's solution and 10% of dextran sulfate. A random-primed ^{32}P-labelled DNA probe (2-15 x 10^6 cpm/ml) was added to the hybridization solution. After hybridization, the membranes were washed 2 times using 0.1X SSC, 0.1% SDS, in a final volume of 20 ml at room temperature for 10 min each, followed by 2 washes in 0.1X SSC, 0.1% SDS at room temperature for 15 min each and finally 2 washes in 0.1X SSC, 0.1% SDS for 30 min each at 65°C. The membranes were then exposed at -80°C to Kodak Biomax films.

Chapter 3

Results

3.1 Detection of phosphorylated proteins by western analysis

Western detection experiments are often used to detect the amount of specific proteins found in the extract. This observation will indicate any variation in the amount of translated material before and after treatment. In this specific study, we used anti-phospho-threonine, -tyrosine and -serine antibodies. They specifically bind to the corresponding phosphorylated residue, thus indicating if any variation in the amount of the specific phosphorylated residue occurred in the experiment.

3.2 Spray-inoculated time-course experiments

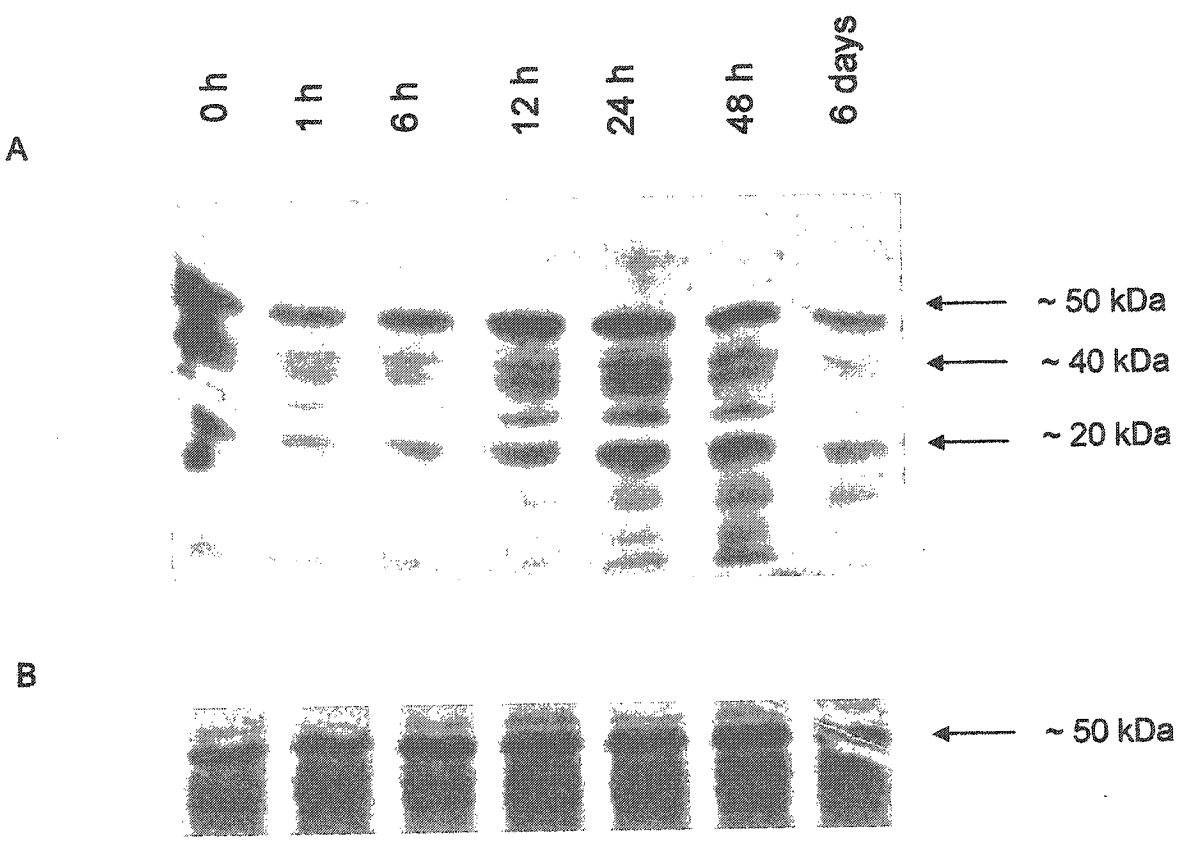
A series of experiments were performed to measure variation in phosphorylated residues following inoculation of wheat heads from cultivars Frontana and Roblin with a fungal suspension of *Fusarium graminearum*. Wheat heads sprayed with water acted as the control. Soluble proteins were extracted from wheat heads inoculated with *Fusarium graminearum*, collected at 0, 1, 6, 12, 24, 48 h and 6 days, and prepared for gel electrophoresis and western analysis as described in Material and Methods. A control western detection experiment was also performed using only the secondary antibody and several protein samples in order to determine if any non-specific binding was occurring. This is shown in Appendix C. The only observable band corresponds to the migration front of the proteins. Thus, non-specific binding was not an issue.

After incubating with the anti-phospho-threonine antibody (Figure 9A), the pattern of phosphorylation of the *Fusarium graminearum*-treated Roblin samples remained constant between 0 and 48 h. The band pattern of this interaction was characterized by intense bands at ~ 50 kDa and ~ 20 kDa, and series of weaker bands (4-5), including a ~ 40, 44 and 47 kDa triplet. Only after 6 days was there a change in phosphorylation, with a noticeable decrease in the intensity of the ~ 40 and ~ 44kDa bands of the triplet as well as a ~ 30 kDa and a ~ 50 kDa band. As shown by the intensity of the ~ 50 kDa band in the Coomassie blue stained gel (Figure 9B), protein loading was relatively constant.

For the interactions between water-treated Roblin, *Fusarium graminearum*-treated Frontana and water-treated Frontana samples, with the anti-phospho-threonine, only 2 lanes for each time-course will be shown. The 1 h sample was chosen as a representative of the 0 to 48 h samples, while the other sample will show changes in phosphorylation pattern, when applicable, in the 6-day sample. This was done in order to limit the repetition of uninformative results consisting of times when no changes in pattern were observed. This was done for all other incubations. Also, the ~ 50 kDa band found on the Coomassie Blue stained gel is shown for every experiment in order to show protein loading.

As a control, wheat heads were sprayed with sterile water and samples were harvested at the same times as for infected heads. Anti-phospho-threonine antibody interaction with extracts from Roblin wheat heads treated with water showed an interesting pattern (Figure. 10A). The 1 h sample showed a band pattern similar to that of *Fusarium graminearum*-treated samples. However, in

Figure 9: A) Western detection experiment demonstrating interaction between anti-phospho-threonine antibody and soluble extracts of wheat heads of cultivar Roblin following infection by spraying with *F. graminearum*. Extracts were prepared from samples collected at 0, 1, 6, 12, 24, 48 h and 6 days **B)** Coomassie blue stained gel of the same extracts as in Figure A showing protein loading.

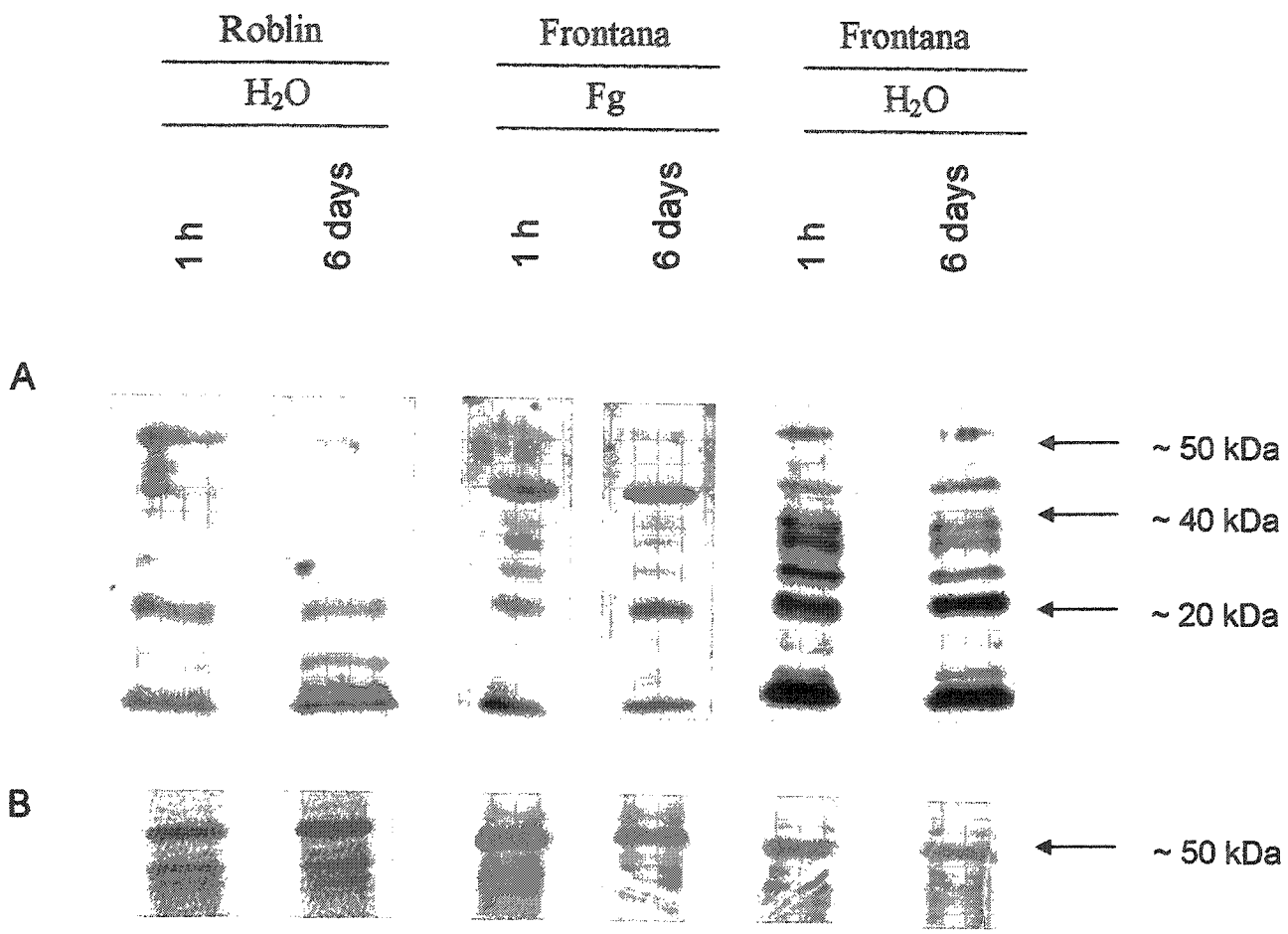

A
0 h
1 h
6 h
12 h
24 h
48 h
6 days
~ 50 kDa
~ 40 kDa
~ 20 kDa
B
~ 50 kDa

the 6-day sample, a change in the phosphorylation pattern occurred when a band of ~ 49 kDa first appeared. The ~ 49 kDa band was not observed in the anti-phospho-threonine interaction with the *Fusarium graminearum*-treated wheat heads (Figure 9A). Of the ~ 40-47 kDa triplet, only the ~ 47 kDa band changed in phosphorylation, as it was absent in the 6-day sample. The apparent change with the ~ 35 kDa band at the 6-day sample was never reproduced. The other differences in phosphorylation intensity pattern were reproduced with the other biological samples.

Also shown is the interaction between anti-phospho-threonine and samples treated with *Fusarium graminearum* taken from the resistant cultivar Frontana (Figure 10A). This interaction revealed a consistent phosphorylation pattern as well as consistent phosphorylation intensity until the 6-day sample. The relative phosphorylation intensity pattern was quite similar to that observed with *Fusarium graminearum*-treated samples from Roblin. These similarities were reproduced with fresh protein extracts.

Finally, the water-treated samples from Frontana showed no detectable/reproducible variation within the phosphorylation intensity and pattern. The band pattern was similar to the Frontana *Fusarium graminearum*-treated samples. The minor differences in phosphorylation intensity changes in the 6-day sample were not reproducible and may be attributed to protein loading. The relative intensity of the bands in the patterns was different between water-treated and *Fusarium graminearum*-treated samples.

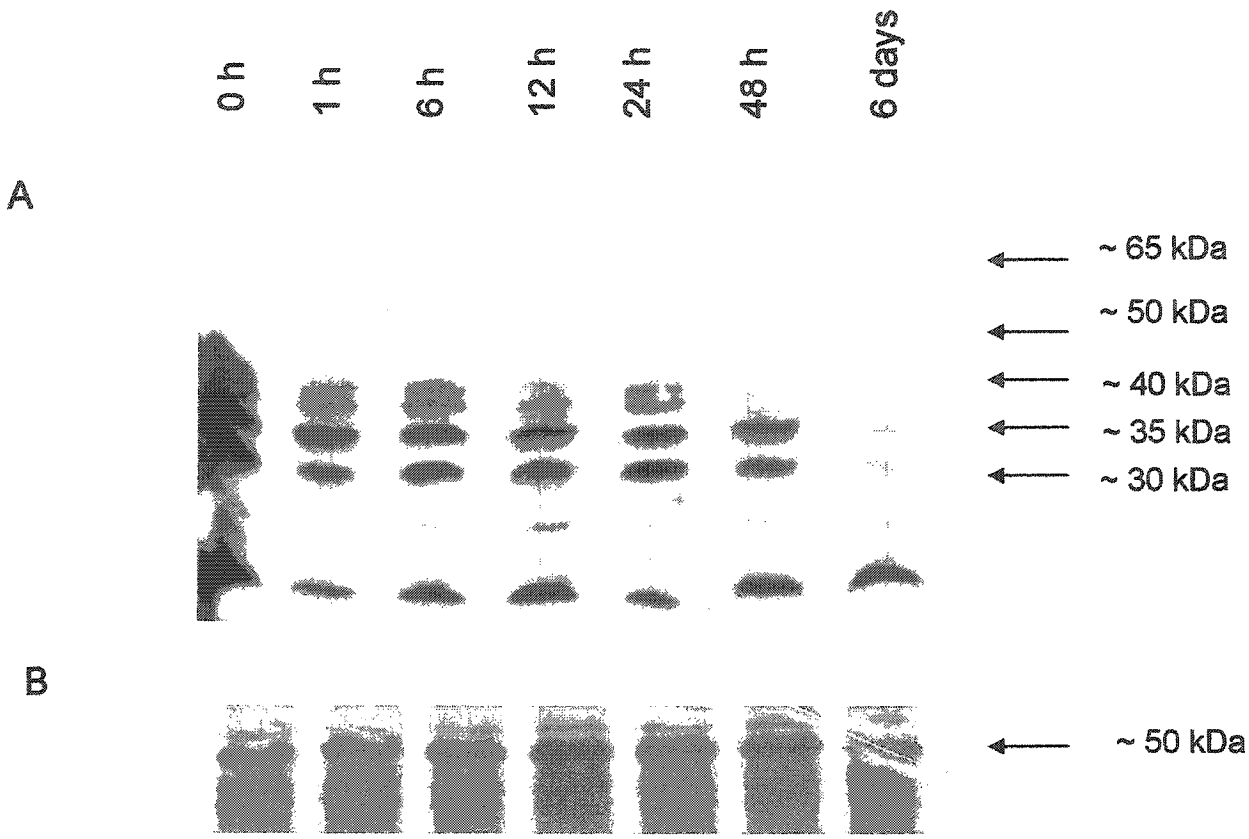
Figure 10: **A)** Western detection experiment demonstrating interaction between anti-phospho-threonine antibody and soluble extracts of wheat heads of cultivar Roblin and Frontana following infection by spraying with *F. graminearum* and water. Extracts shown were prepared from samples collected at 1h and 6 days **B)** Coomassie blue stained gel of the same extracts as in Figure A showing protein loading.


Roblin
H2O
Frontana
Fg
Frontana
H2O
1 h
6 days
1 h
6 days
1 h
6 days
A
~ 50 kDa
~ 40 kDa
~ 20 kDa
B
~ 50 kDa

A different pattern was observed when an anti-phospho-tyrosine antibody was used on *Fusarium graminearum*-treated Roblin samples (Figure 11A). The band pattern was characterized by two intense bands at ~ 30 and ~ 35 kDa in size. The ~ 20 and ~ 50 kDa bands were much lighter in intensity when compared to the anti-phospho-threonine interaction. Also, there were several faint bands from ~ 50 kDa to ~ 65 kDa, which did not appear to vary in phosphorylation intensity, within the time-course. The time-course experiment showed that the pattern and intensity of phosphorylation was similar until the 48-h sample. At this point, a ~ 40-44 kDa doublet, similar to that shown in the anti-phospho-threonine interaction, decreased in intensity and was almost completely absent in the 6-day sample. Two other bands, at ~ 30 and at ~ 35 kDa, also decreased in phosphorylation intensity in the 6-day sample. The ~ 37 kDa band was more noticeable due to the decrease in intensity of the ~ 35 kDa band. The ~ 37 kDa was never shown to increase in phosphorylation intensity in other experiments.

Incubation of the anti-phospho-tyrosine antibody with the water-sprayed Roblin samples was shown in Figure 12A. An intense band at ~20 kDa, bands at ~ 30 and ~ 35 kDa and fainter bands throughout characterized the band pattern. The intensity and the pattern of the phosphorylated residues did not change throughout the time-course, except for the 6-day time period. At this time point, a ~ 47 kDa band clearly increased in intensity, yet was very faint in the other

Figure 11: **A)** Western detection experiment demonstrating interaction between anti-phospho-tyrosine antibody and soluble extracts of wheat heads of cultivar Roblin following infection by spraying with *F. graminearum*. Extracts were prepared from samples collected at 0, 1, 6, 12, 24, 48 h and 6 days **B)** Coomassie blue stained gel of the same extracts as in Figure A showing protein loading.


0 h
1 h
6 h
12 h
24 h
48 h
6 days
A
~ 65 kDa
~ 50 kDa
~ 40 kDa
~ 35 kDa
~ 30 kDa
B
~ 50 kDa

samples. As for the fungus-treated sample, the ~ 40 and ~ 44 kDa bands decreased in intensity in the 6 day sample. There was also a ~ 10 kDa, a ~ 20 kDa and a ~ 32 kDa band which seemed to increase in phosphorylation intensity, yet this was not reproduced in other experiments. The relative phosphorylation intensity pattern of the bands in the time-course was quite different when compared to its *Fusarium graminearum*-treated sample homologue. The *Fusarium graminearum*-treated samples showed intense bands of ~ 30-35 kDa while the same bands found in the water-treated samples were less intense. In the water-treated samples, bands found at ~ 20 kDa and in the ~ 50-65 kDa range were much more intense, than those found in the *Fusarium graminearum*-treated samples. These phosphorylation intensity pattern differences varied from extraction to extraction, and were not observed with different biological samples.

The *Fusarium graminearum*-treated Frontana samples showed a band pattern characterized by intense bands at ~ 20 kDa, ~ 30 kDa and ~ 35 kDa (Figure 12A). There was also a relatively intense band at ~ 60 kDa with fainter bands between. There was a slight change in phosphorylation intensity that occurred in the 6-day sample. This change may be due to uneven protein loading and/or irreproducibility of the transfer process since it was not reproduced. (Figure 12A).

Finally, water-treated Frontana samples also presented a constant level of phosphorylation pattern and intensity (Figure 12A). The band pattern showed intense bands at ~ 20 kDa and ~ 30 kDa with fainter bands throughout. The band pattern also showed several other bands, compared to *Fusarium graminearum*-

treated samples, but this might be due to a better resolution of the SDS-PAGE system. This pattern was similar to that of the *Fusarium graminearum*-treated samples.

When western detection experiments with the anti-phospho-tyrosine or anti-phospho-threonine antibodies were performed, the protein samples were transferred onto a 100% nitrocellulose membrane and casein was added to the blocking buffer. When western detection experiments that used the anti-phospho-serine antibody were performed with this combination of membrane and blocking agent, very high levels of background were observed, probably caused by the presence of serine residues in the blocking buffer. After numerous rounds of troubleshooting, the solution found was to change the hybridization membrane and blocking buffer. Thus for this antibody, samples were transferred onto a nitrocellulose-ester coupled membrane and gelatine was added to the blocking buffer, resulting in a lower background. With the anti-phospho-serine antibody **incubated** with *Fusarium graminearum*-treated samples, the band pattern was also different from the two previous experiments (Figure 13A). The ~ 20 kDa band was much more intense, and there was a span of bands going up to ~ 110 kDa. Bands ranging from ~ 60 to ~ 110 kDa showed less intensity and were less crisp, since they were outside the optimal resolution range of a gel at 12.5% poly-acrylamide. The slight variations in phosphorylation intensity observed around ~ 30 kDa and ~ 80 kDa were not reproduced in other experiments. Therefore no reproducible changes were observed after *Fusarium graminearum* treatment.

Figure 12: **A)** Western detection experiment demonstrating interaction between anti-phospho-tyrosine antibody and soluble extracts of wheat heads of cultivar Roblin and Frontana following infection with *F. graminearum* and water. Shown are extracts taken from samples after 1 h and 6 days of treatment. **B)** Coomassie blue stained gel of the same extracts as in Figure A showing protein loading.

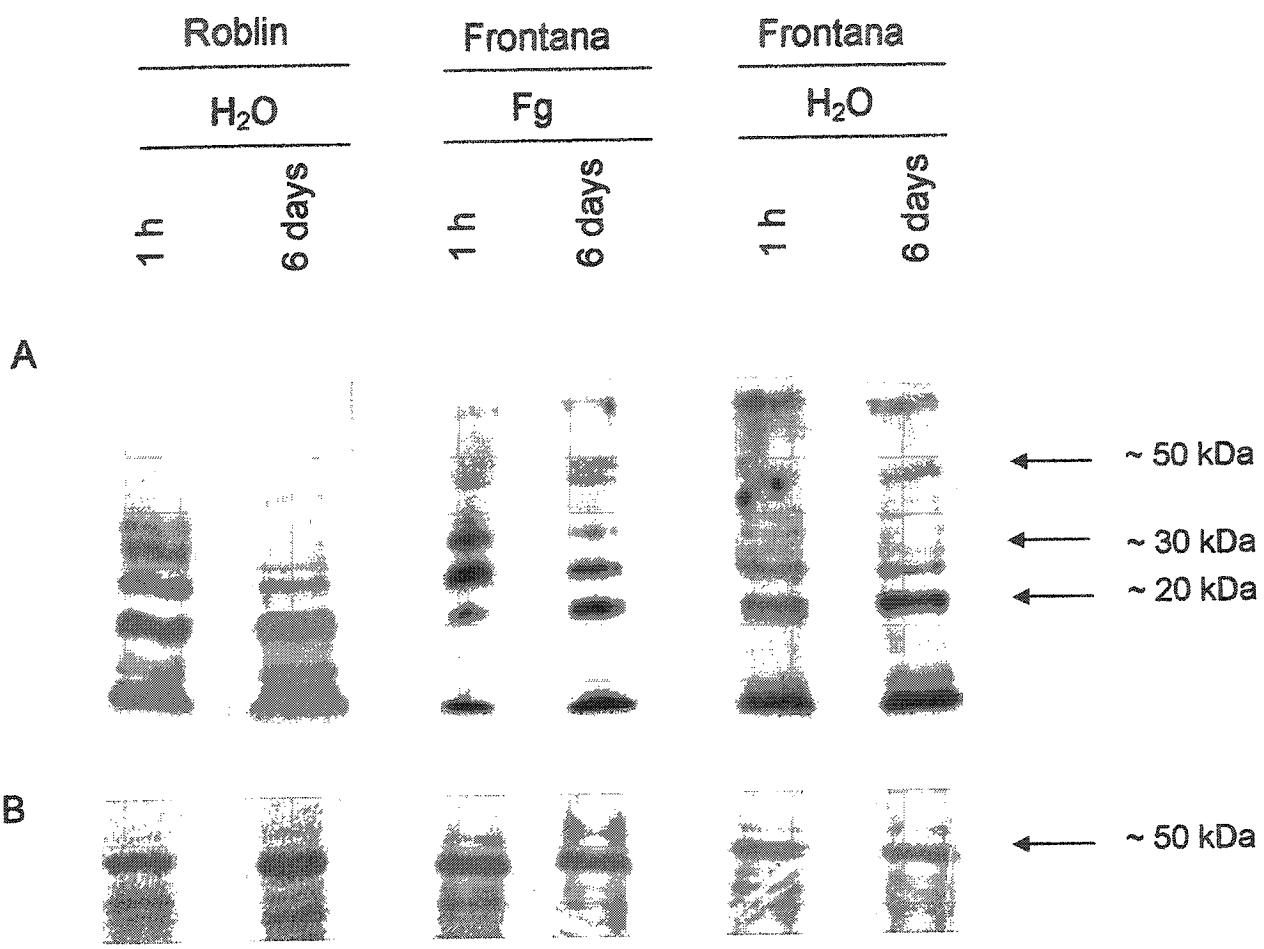
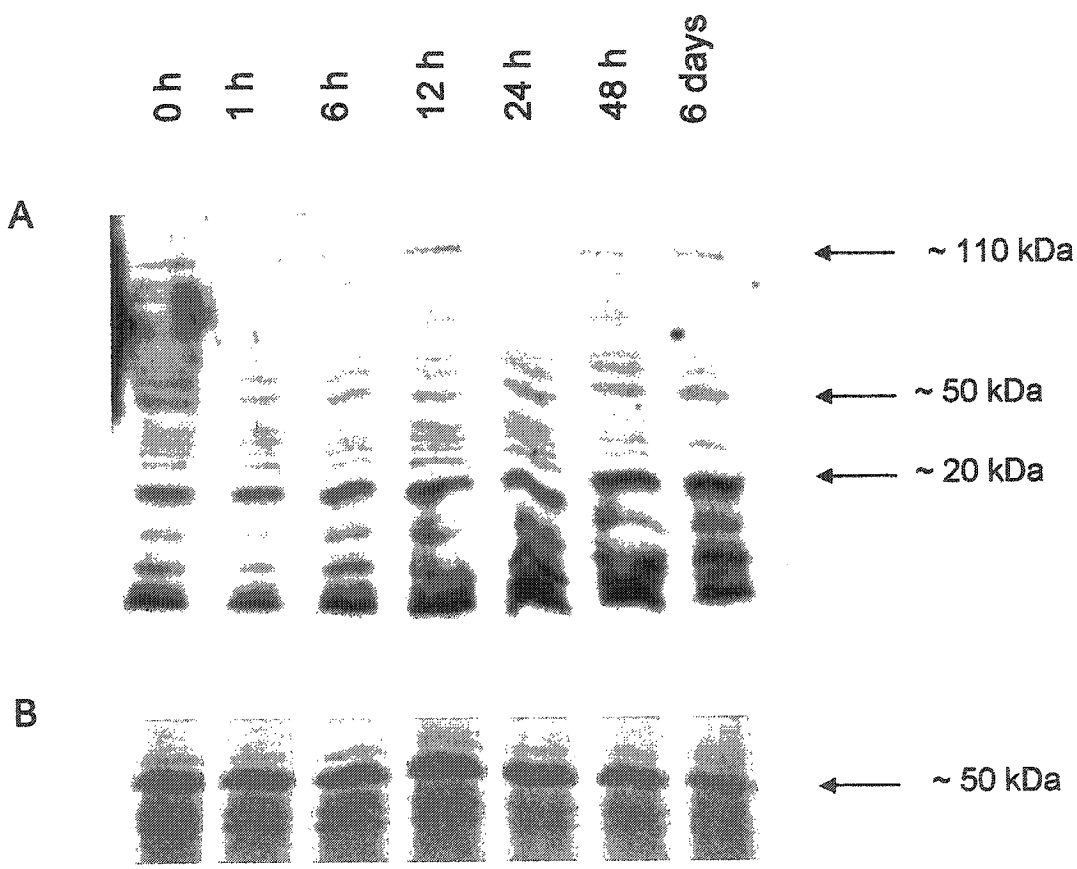

Roblin
Frontana
Frontana
H2O
Fg
H2O
1 h
6 days
1 h
6 days
1 h
6 days
A
~ 50 kDa
~ 30 kDa
~ 20 kDa
B
~ 50 kDa

Figure 13: A) Western detection experiment demonstrating interaction between anti-phospho-serine antibody and soluble extracts of wheat heads of cultivar Roblin following infection by spraying with *F. graminearum*. Extracts were prepared from samples collected at 0, 1, 6, 12, 24, 48 h and 6 days **B)** Coomassie blue stained gel of the same extracts as in Figure A showing protein loading.


0 h
1 h
6 h
12 h
24 h
48 h
6 days
A
~ 110 kDa
~ 50 kDa
~ 20 kDa
B
~ 50 kDa

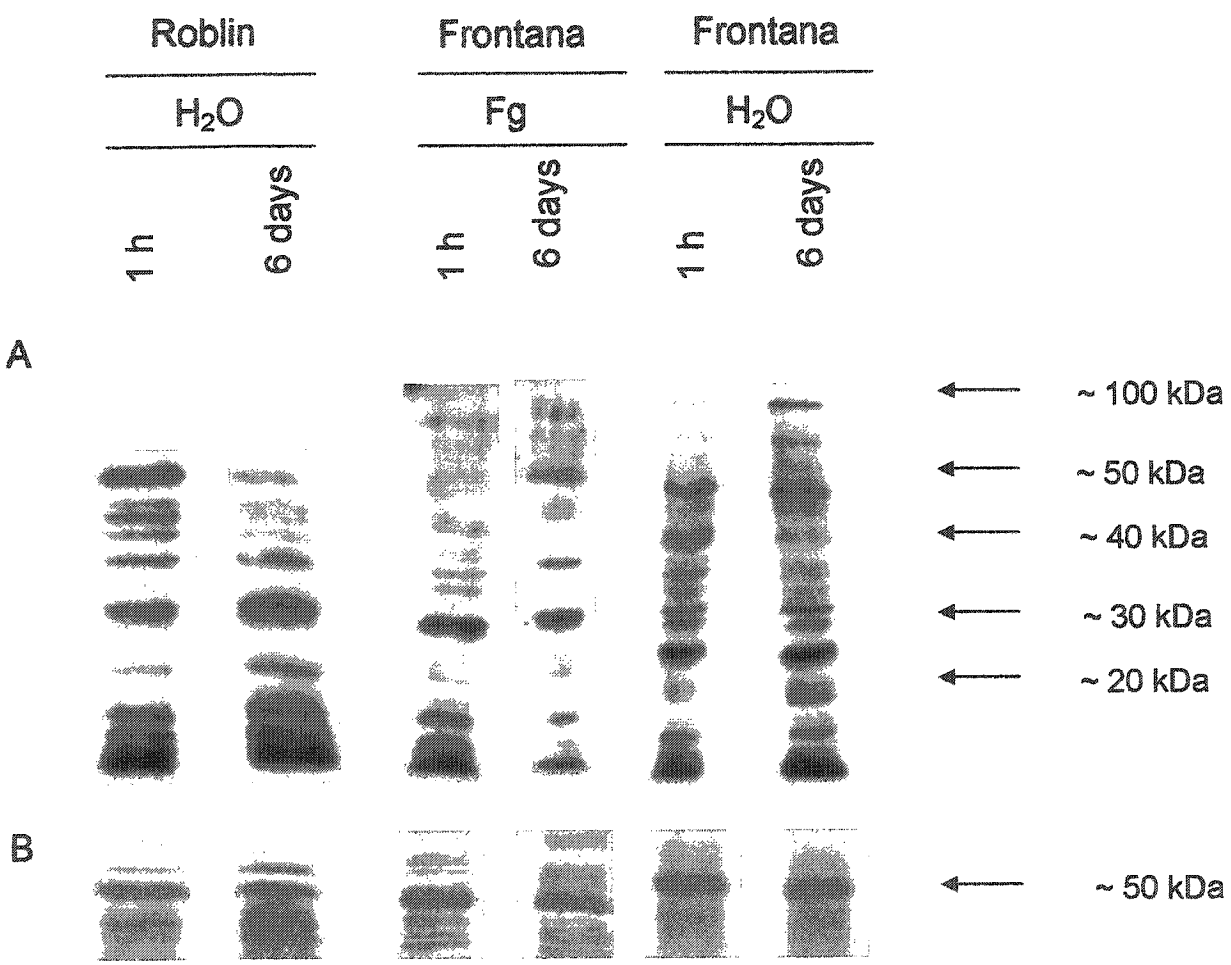
The anti-phospho-serine antibody interactions between the water control samples (Figure 14A) demonstrate a band pattern with intense bands at ~ 30 kDa and ~ 50 kDa. The differences in the presence of bands beyond ~ 50 kDa between treatments were not consistent. A triplet ranging from ~ 44-48 kDa and the ~ 50 kDa band seemed to decrease in intensity in the 6-day sample, yet this was never reproduced. There was also a ~ 30 kDa band that appeared to increase in intensity in the 6-day sample, but again this was never reproduced.

Also, the *Fusarium graminearum*-treated samples of Frontana showed an intense band at ~ 30 kDa accompanied by a wide range of bands up to ~ 100 kDa (Figure 14A). The changes around ~ 35 kDa and ~ 50 kDa in the 6-day sample were not consistent in other experiments.

Finally, the water-treated samples demonstrated a pattern with intense bands at ~20 kDa, ~ 50 kDa, ~ 100 kDa and fainter bands throughout. The intensity patterns between water-treated and fungus-treated samples were generally similar. The slight variation in phosphorylation intensity shown in around ~ 35 kDa and ~ 85 kDa were not reproduced.

Table 2 shows a summary of the changes in band phosphorylation observed at 6-day in the Roblin cultivar. The interaction between anti-phospho-threonine and *Fusarium graminearum*-treated Roblin samples produced a decrease in intensity of ~ 30 kDa and ~ 50 kDa bands, as well as a ~ 40 and ~ 44 kDa doublet. The interaction between anti-phospho-tyrosine and *Fusarium graminearum*-treated Roblin samples showed a decrease in intensity in bands of

Figure 14: **A)** Western detection experiment demonstrating interaction between anti-phospho-serine antibody and soluble extracts of wheat heads of cultivar Roblin and Frontana following infection by spraying with *F. graminearum* and water. Extracts were prepared from samples collected at 1 h and 6 days **B)** Coomassie blue stained gel of the same extracts as in Figure A showing protein loading.


Roblin
Frontana
Frontana
H2O
Fg
H2O
1 h
6 days
1 h
6 days
1 h
6 days
A
B
~ 100 kDa
~ 50 kDa
~ 40 kDa
~ 30 kDa
~ 20 kDa
~ 50 kDa

~ 30 and ~ 35 kDa and also the ~ 40-44 kDa doublet. A similar ~ 40-44 kDa doublet also decreased in the water-treated Roblin samples after incubating with anti-phospho-tyrosine, in addition to an increase in phosphorylation of a ~ 47 kDa band. The interaction between anti-phospho-threonine and water-treated Roblin samples showed an increase in phosphorylation of a ~ 49 kDa band. The anti-phospho-serine interaction did not produce any reproducible changes in phosphorylation intensity in either treatment.

3.3 Point-inoculated experiments

With the use of spray inoculation, the fungus was first in contact with the glume and anthers, before moving inside of the florets then to the remainder of the heads. This raised the possibility that the fungus did not yet infect the rest of the wheat head at sampling time. Point-inoculated experiments were done in order to have more localized areas of infection. The inoculated spikelets were harvested. These contained inner and outer glumes, pistils and anthers. This is in contrast to the spray inoculation, where the whole heads were harvested. By harvesting only the inoculated spikelets, the response to the fungus may be more easily detected. Also, this allowed us to compare methods used to distinguish between type I and II resistance. Type I resistant cultivars are resistant to initial infection while type II resistant cultivars are resistant to the infectious spread within the head. Frontana is known to be a type I resistant cultivar.

Point-inoculated experiments involved inoculating between the inner and the outer glumes of the floret and harvesting the floret after 6 and 24 h, as

Table 2: Summary of phosphorylation changes occurring in the 6-day samples of both *Fusarium graminearum*- and water-treated Roblin cultivar. The intensity of the band of interest was normalized relative to the 50 kDa band observed in the Coomassie-stained gel in order to control for gel-to-gel loading differences. Ratios between the 1 h and 6 d treatment were used to determine the extent of the observed increase or decrease. Reproducible increases of 75% or greater and decreases of 50% or greater are coded by 1. Decreases between 35% and 50% are coded by 2. The direction of the arrow indicates whether an increase (↑) or decrease (↓) was observed.

Band weight	Roblin *Fusarium graminearum*-treated			Roblin Water-treated		
	Threonine	Tyrosine	Serine	Threonine	Tyrosine	Serine
30	2 ↓	1 ↓				
35		1 ↓				
40	2 ↓	1 ↓			2 ↓	
44	2 ↓	1 ↓			2 ↓	
47		absent		absent	1 ↑	
49				1 ↑		
50	2 ↓					

explained in Section 2.2 of the Materials and Methods. In the point-inoculated anti-phospho-threonine interaction (Figure 15A), the band pattern showed intense bands at ~ 50 kDa and ~ 20 kDa and a triplet at ~ 40-47 kDa. When compared to the spray-inoculated band pattern, many similarities were found between patterns. The Roblin 24 h water-treated sample showed a decrease in intensity in the ~ 40 kDa and ~ 44 kDa bands, yet these changes were not reproducible. Another experiment done with the Roblin cultivar gave similar results (data not shown, performed by Antoine Leaustic). In Frontana, a decrease in intensity of the ~ 40 and ~ 44 kDa bands was observed in the 24 h water-treated sample.

The anti-phospho-tyrosine interaction with the point-inoculation samples for Roblin and Frontana showed a band pattern throughout the samples with intense bands at ~ 20 kDa and ~ 30 kDa and very faint bands going up to ~ 65 kDa. The band pattern differed from the spray-inoculated samples in that the number of bands was smaller, and their relative intensity was also different. This is not surprising as the complexity of tissues in the two samples is different.

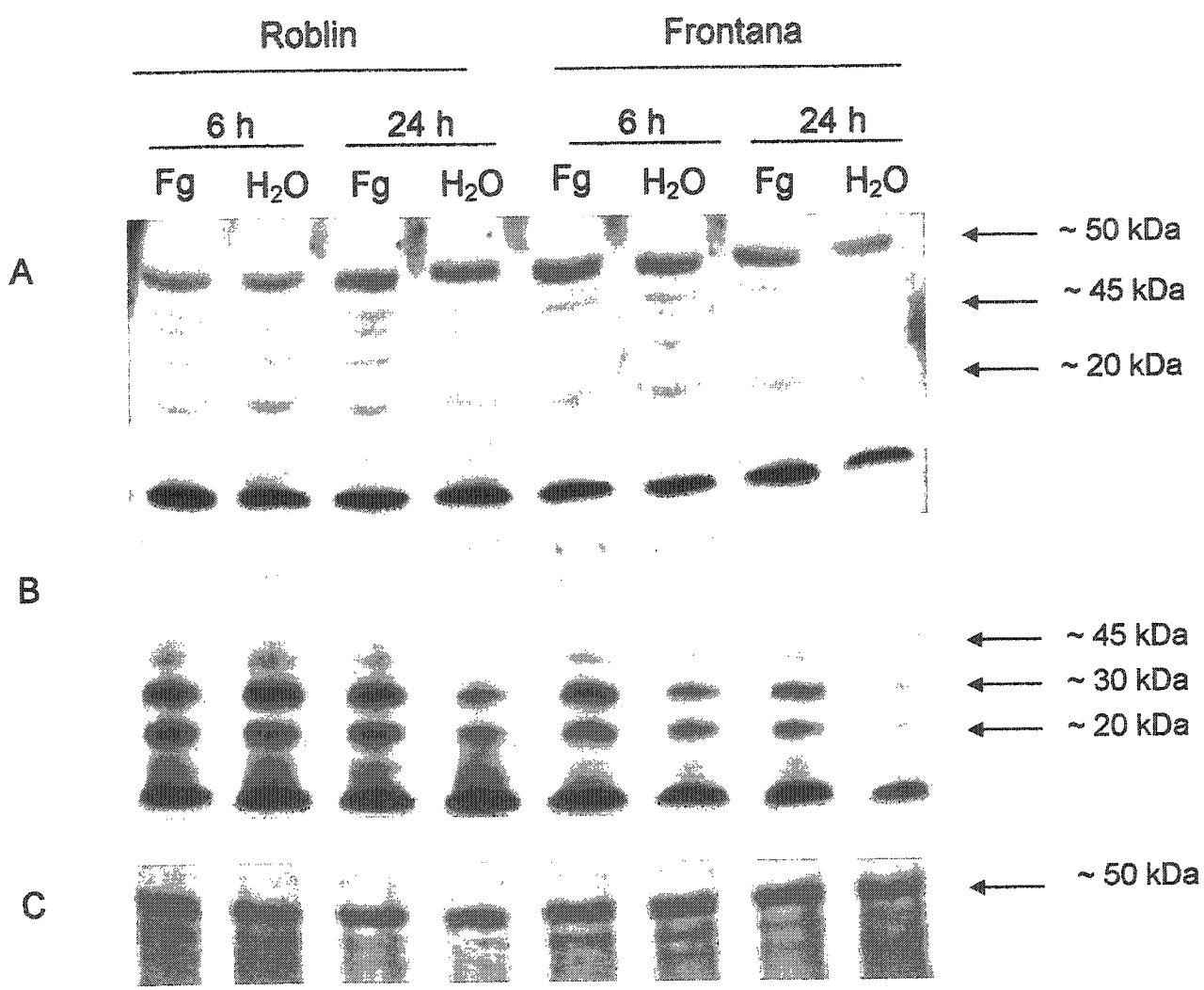
The slight change in phosphorylation pattern found in the 24 h water-treated Roblin sample was not reproducible. Another experiment done on Roblin also showed no variation in phosphorylation intensity (data not shown, performed by Antoine Leaustic). The decrease in intensity of a ~ 40-44 kDa doublet in the Frontana 24 h water-treated sample is important to note. In contrast, the decrease in intensity of the ~ 20 and ~ 30 kDa bands was not reproduced.

In summary, reproducible changes were observed in the ~ 40-44 kDa bands of the water-treated Frontana samples in the interactions with anti-phosho-threonine and anti-phospho-tyrosine.

3.4 In-tube kinase assays

The previous experiments were designed to measure the potential changes in phosphorylated proteins following infection. In-tube kinase assays were performed to measure kinase activities that may play a role in this process. The basis of the in-tube kinase assays is the transfer of the ^{32}P (gamma phosphate of ATP) to an artificial substrate or a natural substrate found in the soluble extract, catalyzed by active kinases in the extract. Several artificial substrates were tested. The results from such experiments should indicate if any active kinase found in the soluble protein extract has phosphorylated the artificial substrate (i.e. MBP (~ 18 kDa), histone III-SS (~ 21 kDa) or casein (~ 23 kDa)), or any natural substrates found in the extract. The spray-inoculated Roblin time series after *Fusarium graminearum* infection was measured with the artificial substrate MBP. This assay did not reveal any phosphorylation activity throughout the time course (Figure 16). The faint line at the bottom of the gel corresponded to ~ 18 kDa, which was similar to the weight of MBP. Yet this also corresponded to the migration front of the protein extract, which contained unincorporated radioactive isotope migrating as low molecular weight material. The acrylamide content was approximately12,5% while the gel in Figure 16 had an acrylamide content of approximately 15%.

Figure 15: Soluble protein extracts of wheat heads of cultivar Roblin and Frontana following infection by point-inoculation with *F. graminearum* and water interacting with **A)** anti-phospho-threonine antibody and **B)** anti-phospho-tyrosine. Extracts were prepared from samples collected at 6 and 24 hrs. **C)** Coomassie blue stained gel of the same extracts as in Figure A showing protein loading.


Roblin
Frontana
6 h
24 h
6 h
24 h
Fg
H2O
Fg
H2O
Fg
H2O
Fg
H2O
A
~ 50 kDa
~ 45 kDa
~ 20 kDa
B
~ 45 kDa
~ 30 kDa
~ 20 kDa
C
~ 50 kDa

Similarly, no phosphorylation activity was observed when histone III-SS and casein were used as substrates on the same *Fusarium graminearum*-treated Roblin samples (data not shown). The Roblin time series after water inoculations also showed no detectable kinase activity with the substrates MBP, histone III-SS and casein (data not shown).

In-tube kinase assays were also performed with Frontana samples. No kinase activity was detected in the *Fusarium graminearum*-treated or the water-treated time course series, with neither of the three artificial substrates (data not shown).

The failures to detect activity with a range of substrates lead us to re-evaluate the in-tube kinase assay system. The positive control used in the in-tube kinase experiments was a recombinant erk2, an active MAPK kinase that demonstrated activity when used with the provided commercial buffer or our sample kinase buffer (data not shown). However, its activity was inhibited when aliquots of wheat head soluble extracts were added to the kinase assay. In Figure 17, we see that kinase activity decreased as the amount of protein extract was increased. Thus, some unknown component or components within the soluble protein extract were inhibiting kinase activity. This result was reproduced with another soluble extract from the same biological sample. The addition of sodium vanadate (0.1 mM), a general phosphatase inhibitor, to the kinase assay reaction mixture was done to verify the possibility that phosphatase activity was responsible for the inhibition observed. Results did not show any reproducible

Figure 16: In-tube assay experiment demonstrating kinase activity found in soluble extracts of wheat heads of cultivar Roblin following infection by spraying with *F. graminearum*. Protein extracts were prepared from samples collected at 0, 1, 6, 12, 24, 48 h and 6 days. MBP was used as artificial substrate and the positive control was an active MAPK, erk2.

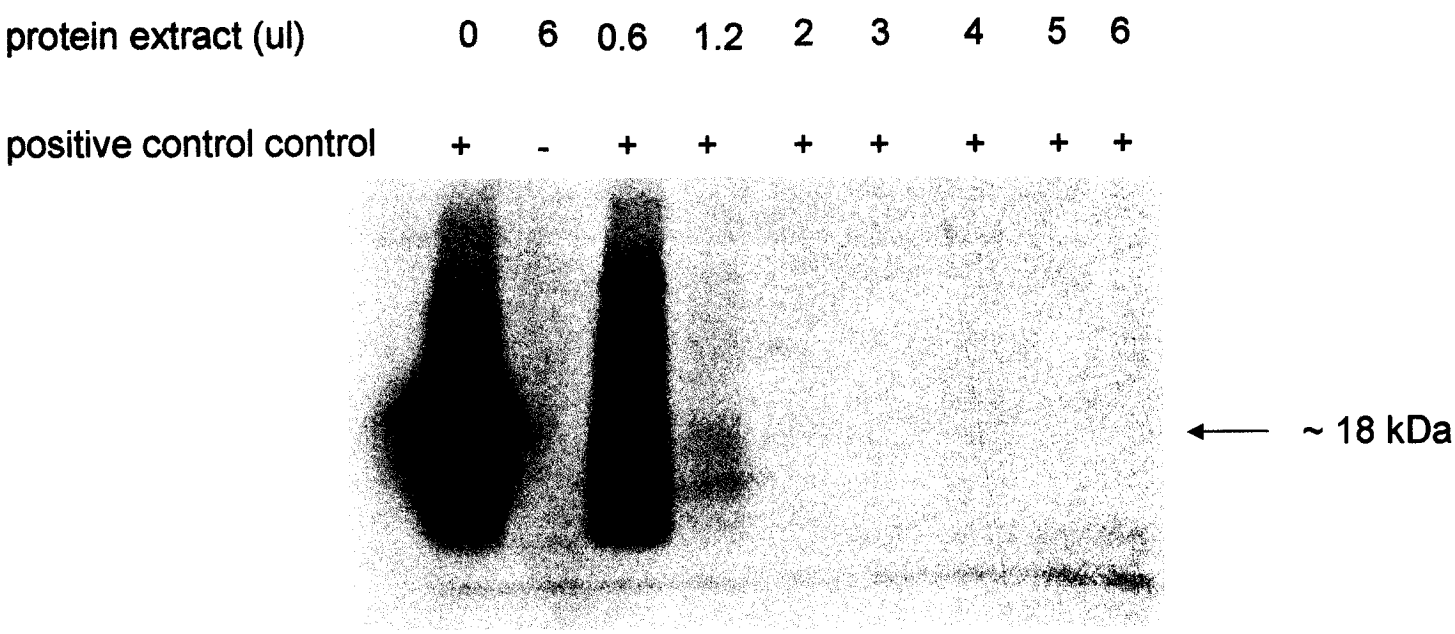
positive control
0 h
1 h
6 h
12 h
24 h
48 h
6 days
~ 42 kDa
~ 18 kDa

changes in kinase activity (data not shown). Thus the in-tube kinase assay cannot be used until the inhibitory component has been removed.

3.5 In-gel kinase assays

For the in-gel kinase assays, the artificial substrate was embedded in the matrix of the poly-acrylamide gel. Labelling would occur only at the migration position(s) where there was an active kinase found, either on the artificial substrate and /or on the kinase itself (autophosphorylation). In contrast, the in-tube kinase assay allowed us to measure the kinase activity on either natural substrate, the artificial substrate found in the reaction or other protein kinases. Therefore, the labelling occurred depending on the substrate that is phosphorylated. In Figure 18, we show an in-gel kinase assay with Frontana and Roblin samples. These samples were extracted from wheat heads spray-inoculated with either *Fusarium graminearum* or water and harvested at 6 and 24 h. Samples from cold-treated corn seedlings of cultivars King Arthur and Seneca were used as positive controls for the procedure. The kinase *ZmMPK5* has been shown to be induced after 3 hours of cold treatment in maize seedlings by Berberich *et al* (1999). Due to the lack of plant tissue for Roblin, only Frontana and some of Roblin samples were assayed. We can see constant kinase activity throughout the wheat head samples for both *Fusarium graminearum*-treated and water-treated samples. The single band may represent a single kinase or multiple kinases having approximately the same molecular size, ~ 45-50 kDa.

Figure 17: In-tube kinase assay using the MBP as artificial substrate with the positive control, erk2. The soluble protein extract was from wheat head of the cultivar Roblin. The series ranges from 0.6 ul to 6 ul of soluble protein extract. The amount of positive control was 0.5 ul.

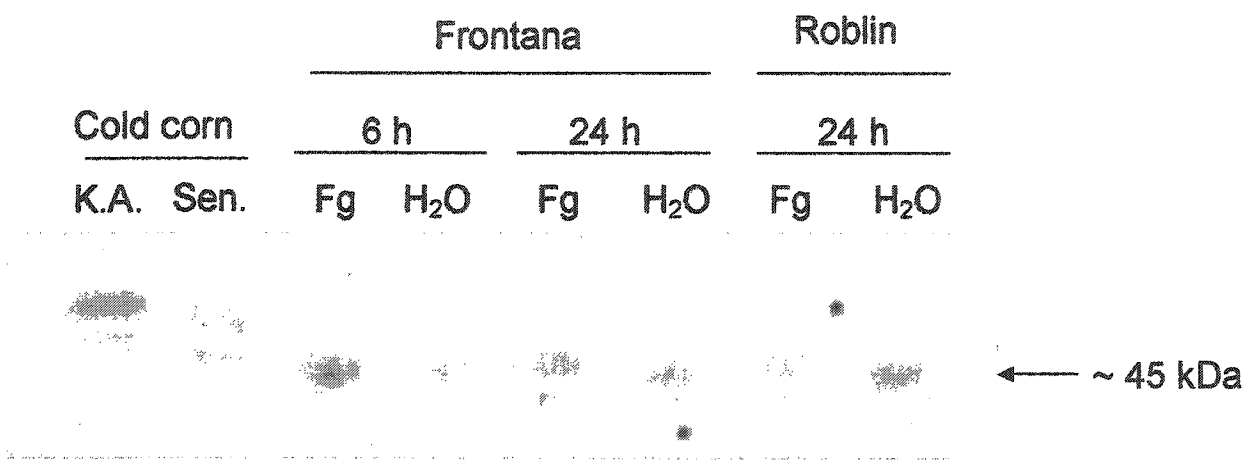

protein extract (ul) 0 6 0.6 1.2 2 3 4 5 6
positive control control + - + + + + + + +
~ 18 kDa

3.6 Isolation of wmh-1

MAPKs have been shown to be involved in several plant defense mechanisms and some of the changes in protein phosphorylation intensity observed previously may correspond to MAPKs implicated in the signal cascade. Therefore the initial cloning strategy aimed to clone a MAPK in wheat. The presence of conserved domains made the choice of primer sites easier and potentially allowed hybridization to as many different sequences as possible.

The strategy used was to isolate total RNA from different cultivars under different treatments in order to clone a transcribed MAPK homologue, and eventually probe differently treated RNA samples from Roblin and Frontana cultivars. Thus we isolated total RNA from Roblin and Frontana after 6 h inoculation with water and *Fusarium graminearum*. The RT-PCR-based approach, as described in section 2.8.3 of Material and Methods, used primers (MKOII and MKDIX) designed to anneal to conserved domains II and IX of MAPKs. The resulting products of approximately 500 bp are shown in Figure 19A. Five clones were obtained and named *wmh-1* to *5*. The clone *wmh-1* was isolated from Roblin 6 h water-treated RNA, while the *wmh-2* clone was isolated from Roblin 6 h *Fusarium graminearum*-treated RNA. Clones *wmh-3* to *5* all came from Frontana 6 h *Fusarium graminearum*-treated RNA. The five clones resulting from the RT-PCR were used for BLAST searches and all showed high similarity to the *wck-1* mRNA sequence found in the public database. The clone identical to the *wck-1* sequence (Takezawa, 1999; accession AF079318) was named *wmh-1* (**W**heat **M**APK **h**omologue-**1**). The five clones were aligned with

Figure 18: In-gel kinase assay experiment demonstrating kinase activity found in soluble extracts of wheat heads of cultivar Roblin and Frontana following infection by spraying with water and *F. graminearum*. Protein extracts were prepared from samples harvested at 6 and 24h. MBP was used as artificial substrate. Positive samples were protein extracts from cold treated corn seedlings from cultivars *King Arthur* and *Seneca*.

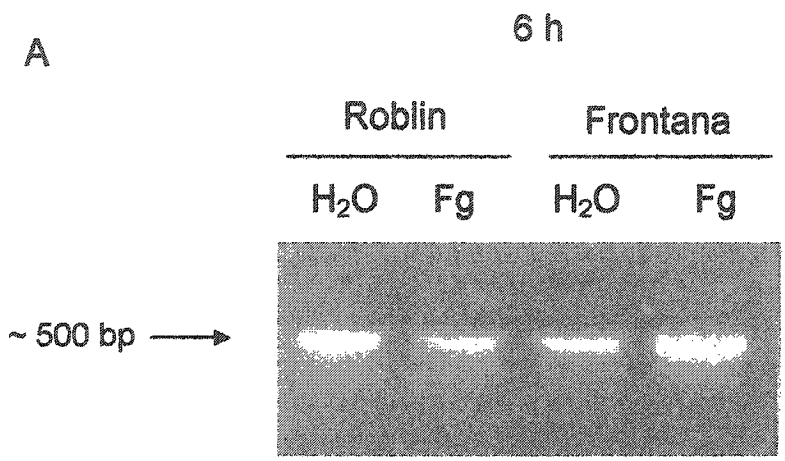

Frontana
Roblin
Cold corn
6 h
24 h
24 h
K.A.
Sen.
Fg
H2O
Fg
H2O
Fg
H2O
~ 45 kDa

the *wck-1* sequence. This alignment is shown in Figure 20. Some of the sequences (*wmh-2, 3, 4*) showed ambiguous bases as indicated by S (C or G) or Y (C or T) indicating that the sequence contained unresolved nucleotides. If we set aside the ambiguous bases and analyzed the remaining sequences, we saw that the changes in *wmh-2, 3* did not affect the amino acid sequence. In *wmh-4*, in position 428, the addition of a G shifts the reading frame, resulting in 3 different amino acids at the end of the sequence. The similarities between sequences allows us to say that a possible interpretation is that *wmh-1* and *wmh-2* are from the same allele in Roblin, while *wmh-4* and *wmh-5* could be found on the same allele, while *wmh-3* would be found on a different allele in Frontana. The alignment shows that the sequences were highly similar with percent identity values between *wck-1* and *wmh-1, 2, 3, 4* and *5* sequences 100%, 98.4%, 97.9%, 95,4% and 98.2% respectively. The similarity of *wmh-1* to the wheat *wck-1* sequence (accession AF079318) was 100% identical (440/440) at the nucleic acid and the amino acid (146/146) levels (Figure 21).

In addition to amplification from cDNA, a PCR reaction was also performed on genomic DNA in order to amplify and identify as many different MAPK homologues as possible. The PCR reaction was performed on DNA extracted from Frontana seedlings, using the same primers as mentioned above. The products are shown in Figure 18B, where 3 replicates are shown. The products' size ranged from 450 to 900 bp. The boxes A, B, C and D indicate the area from which the DNA fragments were isolated. Four clones were picked (*wmh-6* to *9*), however, only one (*wmh-6*, from area C) had some similarity to the

Figure 19: **A)** RT-PCR on RNA samples from Roblin and Frontana treated with water or *F. graminearum* for 6 h. **B)** PCR products using genomic DNA from Frontana as template (1,2 and 3 are replicates). Bands A, B, C and D were isolated and sequenced.

A


6 h
Roblin
Frontana
H2O
Fg
H2O
Fg
~ 500 bp

B

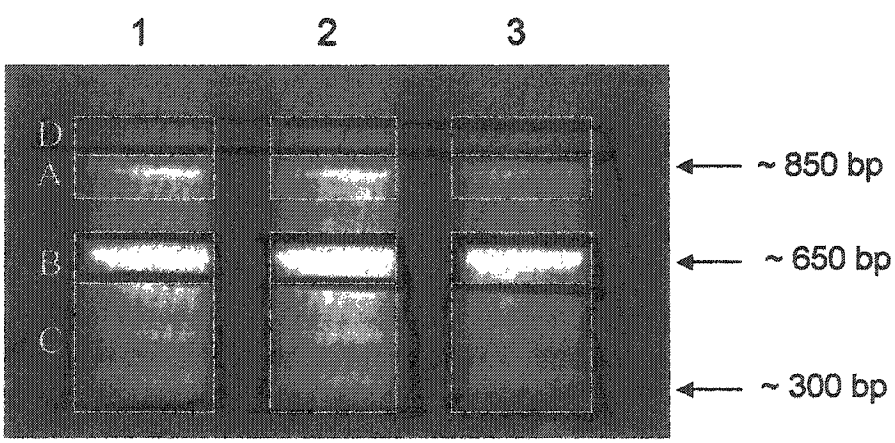

1
2
3
D
A
B
C
~ 850 bp
~ 650 bp
~ 300 bp

Figure 20 : Alignment of nucleic acid sequences of five cloned fragments (*wmh-1* to *wmh-5*) along with the nucleic acid sequence of the *wck-1* clone found in the public database.

319 G C C T T C G A C A A C A A C A T G G A C G C C A A G C G C WCK-1

1 . . . . . wmh-1

1 . . . . . wmh-2

1 . . . . . wmh-3

1 . . . . . wmh-4

1 . . . . . wmh-5

349 A C G C T C C G G G A G A T C A A G C T C C T C A G G C A C WCK-1

31 . . . . . wmh-1

31 . . . . . wmh-2

31 . . . . . wmh-3

31 . . . . . wmh-4

31 . . . . . wmh-5

379 C T C G A C C A C G A G A A C A T A G T A G G C C T C C G A WCK-1

61 . . . . . wmh-1

61 . . . . . wmh-2

61 . . . . . wmh-3

61 . . . . . wmh-4

61 . . . . . wmh-5

409 G A T G T G A T C C C G C C G G C G A T C C C G C A G T C C WCK-1

91 . . . . . wmh-1

91 . . . . . wmh-2

91 . . . . . wmh-3

91 . . . . . wmh-4

91 . . . . . wmh-5

```
439 TTCAACGACGTCTACATCGCCACTGAGCTC WCK-1
121 ............................... wmh-1
121 ............................... wmh-2
121 .C............................. wmh-3
121 ..........................C..... wmh-4
121 ..........................C..... wmh-5

469 ATGGACACGGACCTCCACCACATCATCCGC WCK-1
151 ............................... wmh-1
151 ............................... wmh-2
151 ............................... wmh-3
151 ............................... wmh-4
151 ............................... wmh-5

499 TCCAACCAAGAACTCTCGGAAGAACACTGC WCK-1
181 ............................... wmh-1
181 ............................... wmh-2
181 ............................... wmh-3
181 ..................A............ wmh-4
181 ..................A............ wmh-5

529 CAGTACTTCCTGTACCAGCTGCTGCGCGGC WCK-1
211 ............................... wmh-1
211 ..................S-........... wmh-2
211 ..................S-........... wmh-3
211 ..................S-........... wmh-4
211 ............................... wmh-5
```

559 C T C A A G T A C A T C C A C T C G G C G A A C G T G A T C WCK-1
241 . . . . . wmh-1
240 . . . . . Y . wmh-2
240 . . . . . wmh-3
240 . . . . . wmh-4
241 . . . . . wmh-5

589 C A C C G C G A C C T C A A G C C G A G C A A C C T G C T G WCK-1
271 . . . . . wmh-1
270 . . . . . wmh-2
270 . . . . . G . . A wmh-3
270 . . . . . G . . A . . . . . A wmh-4
271 . . . . . G . . A . . . . . A wmh-5

619 C T G A A C G C C A A C T G T G A C C T C A A G A T C T G C WCK-1
301 . . . . . wmh-1
300 . . . . . wmh-2
300 . . . . . wmh-3
300 . . . . . C wmh-4
301 . . . . . C wmh-5

649 G A C T T C G G C C T G G C G C G G C C G T C A T C G G A G WCK-1
331 . . . . . wmh-1
330 . . . . . wmh-2
330 . . . . . wmh-3
330 . . . . . C . . . . . G wmh-4
331 . . . . . C . . . . . G wmh-5

679 A G C G A C A T G A T G A C G G A G T A C G T G G T C A C G WCK-1

361 . . . . . wmh-1

360 . . . . . wmh-2

360 . . . . . wmh-3

360 . . . . . wmh-4

361 . . . . . wmh-5

709 C G G T G G T A C C G G G C C C C G G A G C T G C T G C T C WCK-1

391 . . . . . wmh-1

390 . . . . . wmh-2

390 . . . . . wmh-3

390 . . . . . wmh-4

391 . . . . . wmh-5

739 A A C T C C A C - C G A C T A C T C C G WCK-1

421 . . . . . - wmh-1

420 . . . . . - wmh-2

420 . . . . . - wmh-3

420 . . . . . G wmh-4

421 . . . . . - wmh-5

wck-1 sequence in the database. The other three clones had only fragments of their sequence homologous to different genomic DNA sequences from rice, yet the lengths of the sequence similar to the rice sequences were approximately 50 bps.

The *wmh-1* sequenced was compared to other wheat kinase EST sequences found in public databases, with the help of computer program developed by Jiro Hattori at ECORC. These ESTs were grouped into contigs according to homology, providing a consensus sequence; the *wmh-1* sequence was aligned with these consensus sequences. The percent homology between *wmh-1* and the different consensus sequences ranged from no significant homology (percent not given by the computer program) to 99% homology. The latter corresponded to the consensus sequence of WCK-1 MAPK homologue ESTs. These sequences were obtained from *Fusarium graminearum*-infected spikes of *Sumai 3*, and from heat stressed flag leaves from the *Chinese Spring* cultivar. Therefore, we may hypothesize that this kinase, *wmh-1*, is not tissue specific, and is present in different conditions of stress. There was also some homology with other MAP kinases, such as MMK2 and a P^{43} MAPK homologue found in various wheat tissues (seedling crowns, roots, leaves, spikes) from different cultivars. These tissues came mostly from either *Chinese Spring* cultivar (susceptible cultivar) or *Sumai 3* (resistant cultivar).

Figure 21: Amino acid sequence alignment of the WMH-1and WMH-5 fragments cloned from wheat heads and the WCK-1 fragment found in the public database. Conserved domains are identified with roman numerals.

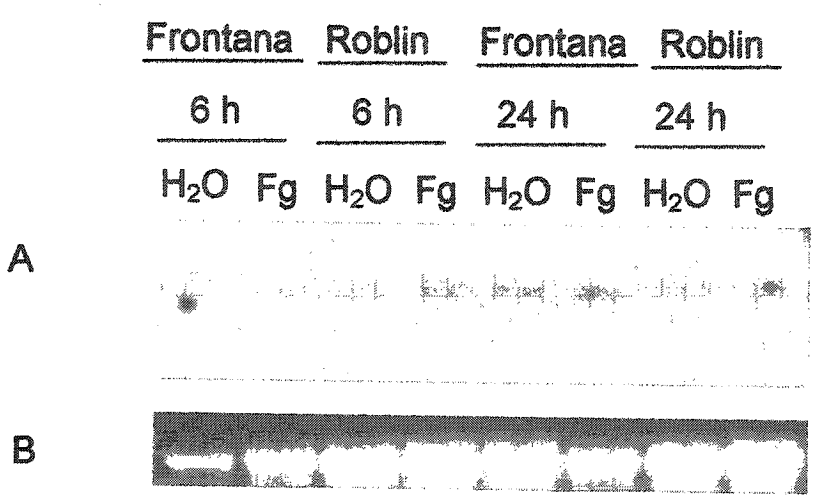
```
                                  III                                IV
 70 AFDNNMDAKRTLREIKLLRHLDHENIVGLR  WCK-1
  1 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  WMH-1
  1 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  WMH-5

                                  V
100 DVIPPAIPQSFNDVYIATELMDTDLHHIIR  WCK-1
 31 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  WMH-1
 31 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  WMH-5


130 SNQELSEEHCQYFLYQLLRGLKYIHSANVI  WCK-1
 61 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  WMH-1
 61 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  WMH-5

      VI                                VII
160 HRDLKPSNLLLNANCDLKICDFGLARPSSE  WCK-1
 91 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  WMH-1
 91 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  WMH-5

                                  VIII
190 SDMMTEYVVTRWYRAPELLLNSTDYS  WCK-1
121 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  WMH-1
121 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  WMH-5
```

3.7 Northern hybridization

Northern hybridization experiments were done in order to measure whether differences in RNA levels could be detected between cultivars or treatments. Thus, if any transcriptional regulation of the *wck-1* gene was occurring due to the presence of the fungal pathogen, the Northern hybridization would indicate such variation. For such an indication, we used the *wmh-1* clone as a probe. The samples consisted of RNA extracted from Roblin and Frontana wheat heads, which have been sprayed with either *Fusarium graminearum* or water, with the tissue harvested after 6 h and 24 h.

As shown in Figure 22A, the variation in intensity of the *wck-1* mRNA was not important after taking into account the variation in loading as indicated by the 25S rRNA subunit (Figure 22B), except for the Frontana 24 h *Fusarium graminearum*-treated sample. There appeared to be an increase in intensity, yet this experiment was been done only once, these are preliminary results and need to be repeated.

Figure 22: A) Northern hybridization with *wck*-1 homologue clone (*wmh-1*) and total RNA from wheat heads. Samples consist of RNA from wheat heads of cultivars Roblin and Frontana after infection by spraying with water and *F. graminearum*. **B)** RNA gel stained with ethidium bromide showing the 25S rRNA subunit in order to show RNA loading.


Frontana
Roblin
Frontana
Roblin
6 h
6 h
24 h
24 h
H2O Fg H2O Fg H2O Fg H2O Fg
A
B

Chapter 4
Discussion

Wheat, a monocotyledonous plant, greatly suffers from infection by *Fusarium graminearum.* The infection causes blighting of the wheat head, as well as reduced yield and quality of seeds. This research project attempted to characterize the wheat response to the fungus with specific emphasis on the possible role(s) of plant kinases during infection. Efforts were concentrated on attempting to measure phosphorylated levels of specific residues, kinase activity and transcriptional levels of a specific MAPK, found in wheat.

In brief we employed several techniques to measure the state of protein phosphorylation in wheat heads including western detection experiments, and in-tube and in- gel kinase assays. Reproducible changes were observed in the pattern of protein phosphorylation in one cultivars, Roblin, following fungal infection with *Fusarium graminearum* as well as in the control treatment only in the latest stage of the time course (6 days). In conjunction with this work, we cloned a homologue of WCK-1 and used it to show that the corresponding level of mRNA expression remained relatively constant in most of the samples. The one exception was an increase in the cultivar 24h after *F. graminearum* infection.

Before considering any of the possible roles of kinases in the pathogen response, a summary of the specific results of this study will be presented. The effects of *F. graminearum* infection on the expression of protein kinases found in wheat heads will be discussed. Finally, the experimental work that could further

this area of research and the possible applications of such research will be presented.

4.1 Summary of results

Initially, there were two time-course experiments designed per cultivar. These time course experiments consisted of spray inoculating the cultivars with either *F. graminearum* or water for 0, 1, 6, 12, 24, 48 h and 6 days. In cv Roblin, the anti-phospho-threonine interaction to both the *F. graminearum*- or water-treated samples were reproducibly different in the 6-day samples. Treatment with *F. graminearum* lead to a decrease in phosphorylation intensity for bands of ~ 30 kDa and ~ 50 kDa, as well as two bands from a triplet at ~ 40 and ~ 44 kDa (Figure 9). The corresponding water-control experiment showed a sudden increase in the intensity of an ~ 49 kDa band and the absence of a ~ 47 kDa band, while the ~ 40-44 kDa doublet did not vary (Figure 10A). In addition, *F. graminearum*-treatment also caused a decrease in intensity of a ~ 40-44 kDa doublet and ~ 30 and ~ 35 kDa bands in the 6-day sample following an incubation with the anti-phospho-tyrosine antibody (Figure 11). At 6 days, in water-treated samples, the ~ 40-44 kDa bands also decreased in intensity while a ~ 47 kDa band increased in intensity (Figure 12A). Finally, anti-phospho-serine antibody interactions with samples from both time course experiments did not show any reproducible changes in phosphorylation intensity or pattern (Figures 13,14A).

F. graminearum infection did not cause a decrease in phosphorylation intensity in the resistant cv Frontana when incubated with the anti-phospho-threonine antibody (Figure 10A). Water-treated samples did not show any reproducible phosphorylation differences with this antibody (Figure 11A). The band patterns and intensity in both *F. graminearum*-treated and water-treated samples following an interaction with either the anti-phospho-tyrosine or anti-phospho-serine antibodies remained constant (Figures 12A, 14A).

The point inoculation experiments showed some reproducible variations in phosphorylation intensities and pattern. With both anti-phospho-tyrosine and -threonine antibodies, the water-treated 24-h Frontana sample showed a decrease in phosphorylation intensity of ~ 40-44 kDa while no changes were observed in the *F. graminearum*-treated samples (Figure 15A, B). The variation in the phosphorylation intensity in the Roblin water-treated 24-h sample, for both anti-phospho-threonine and anti-phospho-tyrosine interactions were not reproducible; therefore, conclusions cannot be drawn at this time from these results (Figure 15A, B).

The in-tube kinase assays proved to be inconclusive as far as the effects of *F. graminearum* on kinase activity are concerned. With all of the artificial substrates used, i.e., MPB, histone III-SS and casein, no kinase activity could be detected (Figures 16). Some preliminary results pointed to presence of either kinase inhibitors or phosphatases in the protein extract, explaining the lack of results (Figure 17). Some kinase activity could be detected with in-gel kinase

assays yet no reproducible variations occurred between *F. graminearum*- and water-treated samples (Figure 18).

Finally, a *wck-1* homologue was cloned from different cultivars following fungal or water treatments. This was the only MAPK that could be detected and/or cloned from total RNA and genomic DNA. The *wmh-1* clone was identical to the nucleic acid and protein sequences of the wheat *wck-1* gene, characterized by Takezawa (1999) and found in the public databank (ncbi.nih.nlm.gov)(Figures 20, 21). Northern hybridization performed with this *wck-1* homologue (*wmh-1*) showed an increase in intensity in the Frontana 24-h *F. graminearum*-treated sample. These are preliminary results that need to be repeated (Figure 22).

4.2 Western detection experiments

4.2.1 Spray inoculations

Protein phosphorylation has proven to be an effective mode of regulation of protein activity. Protein kinases have been shown to phosphorylate specific residues in order to activate proteins (reviewed by Jonak *et al.* 1994). The involvement of threonine, tyrosine and serine in kinase activation are well documented. The western detection experiments using specific anti-phospho-threonine, -tyrosine and -serine antibodies gave the opportunity to search for phosphorylated sites on a diverse group of proteins found in the soluble protein extract of wheat heads.

The water-treated time course experiment gave us the naturally occurring phosphorylation pattern following a slight mechanical stress (handling of the wheat heads). Therefore, any reproducible variation in phosphorylation occurring after fungal-treatment must differ from this standard. The incubations with both anti-phospho-threonine and anti-phospho-tyrosine antibodies presented some differences between the water-treated samples and the *F. graminearum*-treated Roblin samples. The differences occurring in the interaction with anti-phospho-threonine consisted in changes in phosphorylation intensity of several bands but only in the 6-day sample. More specifically, 4 bands with molecular weights of ~ 30, ~ 40, ~ 44 and ~ 50 kDa decreased in intensity. In contrast, the water-treated 6-day sample incubated with anti-phospho-threonine showed an increase of a ~ 49 kDa band, which was not observed during *F. graminearum* infection. Therefore, a possible interpretation is that the fungus caused a decrease in the phosphorylation of threonine residues found on 4 proteins with molecular weights of approximately ~ 30, ~ 40, ~ 44 and ~ 50 kDa. The lack of increased intensity in the ~ 49 kDa band can be attributed to the presence of the fungus. Thus several proteins, and perhaps more that are not detectable by this method, demonstrated reduced phosphorylation of threonine residues.

The interaction with the anti-phospho-tyrosine also showed reproducible results. The fungus-treated 6-day sample showed a decrease in four different bands: ~ 30, ~ 35, ~ 40 and ~ 44 kDa. In contrast, the water-treated 6-day sample shows a decrease in intensity in the ~ 40 and ~ 44 kDa bands while a ~ 47 kDa band increases in intensity. Therefore, we suggest that the fungus

causes a decrease in phosphorylation of tyrosine residues found on proteins of molecular weight ~ 30 and ~ 35 kDa and may be responsible for the absence of increased phosphorylation of the ~ 47 kDa band in the 6-day sample.

The resolution of the gel system allowed us to compare phosphorylation patterns between gels. This was done with the protein molecular markers as reference points. The speed of the method was conducive to a better and more consistent gel resolution.

In retrospect, we can suggest that the ~ 30 kDa band found in both anti-phospho-threonine and anti-phospho-tyrosine interactions could be the same protein. If so, this protein would be regulated by the level of phosphorylation of both types of residues, 6 days after the initial infection with *F. graminearum*. The same argument could be made for the ~ 40 and ~ 44 kDa proteins except that the tyrosine residues found on the protein are not dephosphorylated due to the presence of the fungus. Regulation would still be achieved through the dephosphorylation of threonine residues found on the protein in question. An alternative, the possibility is that there are multiple proteins having the same molecular weight migrating at the same point; therefore the residues could be on separate proteins.

No reproducible variations in phosphorylation intensity in any experiment were observed when the anti-phospho-serine antibody was used. This result was unexpected. The phosphorylation pattern for the anti-phospho-serine antibody interaction was expected to have similarities to the pattern observed with the anti-phospho-threonine antibody since it was anticipated that serine/threonine

kinases, which are a major kinase family necessary for the activation of protein kinases and other proteins (Hardie, 1999), would be implicated in regulation.

Several factors may contribute to the lack of measurable variation in the phosphorylation intensity of serine residues. First, the assay system may lack sufficient sensitivity for the detection of these changes. Second, different types of membrane were used during the transfers for anti-phospho-serine (see section 3.2 of Results). The implication of this observation is that when comparing experiments with different antibodies, one cannot directly relate the experiments occurring on different types of membranes as each type of membrane has specific properties, which affect protein binding. Therefore caution is needed when directly comparing different analyses of the effects of serine/threonine kinases unless the same type of membrane is used. Lastly, the phosphorylation of specific residues on the protein may not be equally important or distributed. Some residues may play more important roles in the activation of the protein in question. This is the case with the OsBIMK1 kinase in rice; this MAPK homologue must be activated by phosphorylation at the TEY (threonine, glutamic acid, tyrosine) (Song and Goodman, 2002). Therefore, it is possible that proteins activated during the time-course experiments (either water or *F. graminearum*) did not require the phosphorylation of serine residues for their activation.

In Frontana, no reproducible changes were observed following incubation with anti-phospho-threonine. The lack of reproducible variation in phosphorylation intensity with other antibodies may reflect differences in susceptibility for each cultivar to the fungal pathogen. Western experiments were

performed on extracts from the cultivar Frontana in order to compare the response to infection between susceptible and resistant cultivars. The fact that Frontana did not show any changes in phosphorylation illustrates the difference in response between Frontana and Roblin cultivars. Frontana did not show many differences between fungal- and water-treated samples, indicating a higher resistance to spray-inoculations with *F. graminearum*. There may be many possible reasons for the different response observed in the two cultivars. They may be attributed to physical characteristics found on the Frontana cultivar that are absent on the Roblin cultivar. The resistance may be due to proteins constitutively expressed in the wheat tissue, or a larger range of proteins involved in different defense mechanisms. These proteins may range from receptors, to signal proteins to chitinases and glucanases, increasing the plant's defense response.

The water-treated samples did not show any reproducible variation in phosphorylation intensity as compared to Roblin. This might indicate that the two cultivars were not at the same maturation point when came time to harvest, even though the two cultivars were at the same developmental stage at the time of inoculation. The maturation process of Frontana is approximately 2-3 weeks longer than that of Roblin (G.Fedak, ECORC, personal communication). The maturation process of the wheat head may also be a cause of phosphorylation variation. Research on the role(s) of protein kinases in plant maturation has demonstrated the presence of a pollen-specific, calcium-dependant and calmodulin-independent protein kinases involved in germination in maize

(Estruch, *et al*, 1994). The multiple roles of receptor-like kinases have also been associated with plant development (reviewed by Becraft, 2002). For example, the presence of RTK1-2-3 in the developing endosperm of *Zea mays* was observed by Li and Wurtzel (1998).

When all the results are analysed together, several explanations may be suggested. First, the presence or activation of a dephosphorylating agent, (i.e, phosphatase) may explain the decrease in phosphorylation of specific residues. Phosphatases have been shown to play important roles in signal transduction in plants. Phosphatases with dual-specificity, tyrosine and serine/threonine phosphatases can dephosphorylate either of the phosphorylated residues in the TxY motif, therefore, acting as negative regulators of MAPKs (reviewed by Keyse, 2000). Evidence has been provided that the MAPK type 2C phosphatase MP2C directly inactivates SIMK, a MAPK activated by wounding in alfalfa (Meskiene *et al*, 2003) through dephosphorylation of the threonine residue in its conserved TEY motif. In *Arabidopsis* tyrosine phosphatases are induced 24 h after the plant has been subjected to either cold or salt stresses. The phosphatase activity regained normal levels after 48 h of salt stress (Xu *et al*, 1998). In our studies, the observed reduction in phosphorylation of the targeted residues may be due to increased phosphatase activity due to fungal infection or by decreases phosphorylation or both. Therefore, the possible presence of phosphatases in the protein extract could have as effect the direct dephosphorylation of the protein(s) observed on the gel, or the

dephosphorylation of a kinase, which leads to the decrease in phosphorylation of the observed protein of the gel.

Secondly, the decrease in phosphorylation intensity shown may be caused by the presence of mycotoxins in the infected wheat tissue. *F. graminearum* produces the mycotoxin deoxynivalenol (DON), which readily penetrates the lipid bi-layer and inhibits protein synthesis (Rousseaux, 1988). This inhibition is manifest on several levels: inhibition of initiation, inhibition of elongation and inhibition of termination, or inhibition of all three processes by the inhibition of the peptidyl transferase (Rousseaux, 1988). The amount of mycotoxin present increases throughout the time of the experiments as the fungus spreads (Reid *et al*, 2000), therefore, the decrease in phosphorylation intensity may be associated to an inhibition of the wheat peptidyl transferase. This protein synthesis inhibition may also be closely related with resistance, where a resistant cultivar may be less affected by the fungus, and in turn will accumulate less mycotoxin. Roblin has been shown to be very susceptible to spikelet infection, e.g., when Roblin spikelets were point-inoculated, 87% of spikes demonstrated symptoms of infection. When the same experiment was done on Sumai3 (a resistant cultivar), only 10% of spikes showed symptoms of infections (Okubara *et al*, 2002).

The band pattern in some of the western detection experiments showed a large variety of bands ranging from 10 kDa to 120 kDa. Is this a pattern, which should be expected from soluble protein extracts isolated from total wheat heads? The presence of bands ranging from ~ 5-10 kDa to ~ 43 kDa was shown

in thylakoid membranes of pumpkin leaves, using an anti-phospho-threonine antibody, (Rintamaki *et al*, 1997). Thus, when phospho-threonine antibody western detection experiments were performed on the soluble protein extract of whole heads of wheat, it was not surprising to see more bands in a wider protein size range because the samples consist of whole tissue, with all of the proteins necessary for function and development. Also, it is known that protein phosphorylation and dephosphorylation regulate numerous biological processes, and are catalyzed by protein kinases and phosphatases respectively (reviewed by Hunter, 1995). Therefore it should be expected that numerous bands appear on the western detection experiments of soluble protein extracts from total wheat heads.

In summary, the western detection experiments have demonstrated some differences related to the cultivar and its susceptibility level. Also, some bands were shown to be responding to the presence of the fungal pathogen. The decrease in phosphorylation of bands ~ 30 and ~ 50 kDa after interaction to the fungus-treated Roblin samples with the anti-phospho-threonine may be attributed to the presence of the fungus. The changes observed with the bands ~ 30, ~ 35 and ~ 47 kDa in the fungus-treated Roblin samples incubated with anti-phospho-tyrosine may also be caused by the presence of pathogen. Phosphorylation of serine residues seems to have been unaffected by the absence or presence of the fungus. There also seem to be cultivar specific responses, seen by the lack of reproducible variations in the interaction with Frontana samples. This may be direct result of the difference in susceptibility between the cultivars.

The response time following infection by a pathogen depends on the system in the experiment is conducted. Research on fungal development of *F. graminearum* on wheat spikes performed by Pritsch *et al*, (2000), found that within the first 48-76 h post-inoculation the fungus had penetrated the stomata, exhibited subcuticular growth, colonized glume parenchyma cells and sporulated. This timing was identical between resistant cultivar Sumai 3 and susceptible cultivar Wheaton. They also showed that transcripts from 6 defence genes accumulated as early as 6 to 12 h post-inoculation and peaked at 36 to 48 h post-inoculation. These 6 genes consisted of peroxidase, PR-1, PR-2 (β-glucanase), PR-3 (chitinase), PR-4 and PR-5 (thaumatin-like protein). Finally, greater accumulation of transcripts of PR-4 and PR-5 was observed in Sumai 3, compared to Wheaton. It was also observed in cultured cell lines (cultivar Chihokukornugi) that fungal elicitors taken from snow mold (*Tiphula ishikariensis*) caused a Ca^{2+}- mediated increase in transcript levels of a MAPK homologue, *wck-1* (Takezawa, 1999). The increased transcript levels were observed after 20 min of incubation with the fungal elicitors. The autophosphorylation activity of WCK-1 was induced 10 min after the cells were incubated with the fungal elicitors.

4.2.2 Point inoculations

One of the purposes of point-inoculating wheat heads was to enrich the wheat samples in infected tissue and in turn increase the chance of observing a potential response to the fungal pathogen. The point-inoculation experiment also

showed a different tissue profile; since the rachis was not harvested changes in phosphorylation in proteins found in this tissue are not detected. Point-inoculations were done by inoculating near tissue where the fungus grows rapidly (i.e. ovaries). The fungal inoculum was added into florets, between the inner and the outer glumes and only the tissue directly affected by the inoculum was harvested. Furthermore, spray and point-inoculations were used to distinguish between type I and type II resistance. As discussed in the Introduction, resistant cultivars can be grouped according to type of resistance: type I are resistant to the initial infection, type II are resistant the infectious spread of the pathogen, type III degrade mycotoxins at a faster rate and type IV are tolerant to higher levels of mycotoxins. A comparison of the phosphorylation levels in samples from spray and point inoculations was of interest to us since Frontana shows resistance when spray-inoculated and not when point-inoculated.

The point-inoculations showed a reproducible change in phosphorylation intensity and pattern in the Frontana 24-h water-inoculation sample. This change in phosphorylation may have occurred in the spray-inoculation experiment but the signal may have been diluted by the presence of uninfected tissue was too faint to detect. On the other hand, this could be associated with the differential response of Frontana to both inoculation techniques. It is possible that the point-inoculation technique causes a natural decrease in phosphorylation through handling, and that this decrease was cancelled by the presence of the pathogen, which caused an increase in phosphorylation of threonine residues and tyrosine residues on bands of ~ 40 and ~ 44 kDa in size. The differences in the overall

phosphorylation patterns between the fungal- and water-treated samples from the point and the spray inoculations were reproducible. Thus, we saw a noticeable change in response between the two inoculation methods. The first 6 h after inoculation did not seem to vary between techniques. Therefore, the point-inoculation may have had an effect on the cultivar Frontana 24 h after the inoculation, while the spray-inoculation did not seem to induce a change in phosphorylation throughout the time-course.

Genetic mapping experiments in wheat have provided evidence that 3-4 quantitative trait loci (QTL) are associated with type II resistance to fungal invasion by *F. graminearum* (Ban and Watanabe, 2001). Mapping data have not been published yet for type I resistance, as in Frontana, however breeding efforts have also suggested a multi-genic trait. Therefore, a gene-for-gene phenomenon is unlikely to be present with *Fusarium* head blight. The intrinsic differences between the mechanisms of resistance in the two cultivars, and the differences between these experiments and those in the literature, make it difficult to come to broad conclusions.

4.3 In-tube kinase assays

The in-tube kinase assay allows the observation of active kinase phosphorylating natural substrates, including kinases, found in the protein extract or phosphorylating artificial substrates provided within the experimental protocol. These experiments did not detect any kinase activity in Roblin or Frontana samples. The assay system did not function as expected and when repeated with

erk2, an active recombinant kinase, no measurable kinase activity was detectable if erk2 was mixed with plant extracts. Several interpretations could explain this effect. For example, there could be active phosphatases in the soluble extract, which could dephosphorylate and inactivate the native and introduced kinases found in the reaction mixture, or their substrates following phoshorylation. Active proteases, which could degrade active kinases and/or substrates, may be found in the extract. Consequently, any active kinase naturally found in the protein extract may have been inactivated, or its corresponding substrates dephosphorylated, by any of the components mentioned above or other chemical substances, which could inhibit kinase activity. It is also possible that several of these factors acted to inhibit kinases or that there was no kinase activity in the protein extracts.

The addition of sodium vanadate, a general phosphatase inhibitor, to the kinase assay reaction mixture did not have any effect on the results. Technical improvements such as inhibiting any phosphatases by the addition of multiple phosphatase/protease inhibitors or partially purifying extracts prior to assaying should be conducted.

There is a possibility of a carry-over of SDS from the protein extraction to the kinase assay. This carry-over may inhibit some kinase activity, due to its denaturing property. However, kinase activity was measured using a similar protein extraction buffer, containing SDS. A 46-kDa MAPK was found to be activated by cutting (Usami *et al*, 1995). The amount of SDS present in our kinase assay reaction mixture, is approximately 2%, where the 3X SDS sample

buffer, which stops the kinase reaction, contains 6% SDS. This being said, it is not possible to dismiss the putative effects of SDS, yet as previously observed in other papers, some kinase activity was present. In addition, the kinase activity measured in the in-gel kinase assay is also an indication that kinase activity was not completely quenched by the presence of SDS.

Also the possibility of chemical compounds inhibiting protein kinase activity exists. For example 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), a hydroxamic acid from wheat has been shown to inactivate papain(Perez and Niemeyer, 1989). Cysteine and serine residues on papain were significantly modified by DIMBOA. However the presence of DTT partially restored enzymatic activity. The majority of the research is conducted on maize and wheat seedlings, however 4-acetyl-benzoxazolin-2-one (4-ABOA) has been isolated in corn kernels (Fielder *et al*, 1994). This compound demonstrates fungicide and insecticide properties in corn (Miller *et al*, 1996). In addition, the amount of DIMBOA in different types of tissues and at different stages has been characterized. The amount of DIMBOA in roots and aerial parts of maize seedling shows a continuous decrease after germination (Cambier *et al*, 2000). Thus, the amount of DIMBOA in the wheat heads could be very small, if not negligible.

4.4 In-gel kinase assays.

The in-gel kinase assay measures active kinase that phosphorylates an artificial substrate embedded in the poly-acrylamide gel. The technique is

frequently used when the protein of interest is easily isolated and concentrated, for example Zhang and Klessig (1997) used it to characterize SIPK activity.

Preliminary results used extracts from cold-treated corn as positive control to validate the assay. A ~ 45 kDa band was present in all samples from Frontana and Roblin. The intensity of this band did not change following different treatments nor with different cultivars. The size observed (~ 45 kDa) corresponds to the range of expected size for MAPKs. Some have been identified at 42 kDa (Xiong and Yang, 2003), while others have been identified at 48 kDa (Zhang and Klessig, 1997).

4.5 Northern hybridizations

Previous studies in wheat had found that *wck-1* mRNA was induced by elicitors derived from *Typhula ishikariensis*, suggesting a putative role in plant defence response (Takezawa, 1999). Five of the six clones generated in this study showed homology to the *wck-1* sequence found in the public database (www.ncbi.nih.nlm.gov). The homology of the cloned sequences to the wck-1 is quite high, however differences among them allowed us to segregate them into cultivar-specific groups. Within these groups, one can also see differences, which may reflect the presence of alleles found in the hexaploid wheat. This analysis contains one flaw, that the clones were only sequenced once, and a more thorough analysis would need several rounds of sequencing to confirm these results.

A northern hybridization was conducted with the one clone that showed complete homology to the *wck-1* sequence used as a probe. Samples consisted of Frontana or Roblin wheat heads treated with either *F. graminearum* or water for a 6 and 24 h. Preliminary results of this hybridization showed that the transcript levels of the *wck-1* homologue did not change within the time frame, except for the 24 h-*F. graminearum* Frontana sample. Before any comparisons can be made between the northern hybridization and the in-tube or in-gel kinase assays, one must be aware that the assays examined many different kinases, while the northern hybridization only looked at a specific kinase. Thus, preliminary observations suggest that the transcriptional regulation of *wck-1* was only affected in Frontana, 24 hours after spray inoculation with *F. graminearum*. The surprising aspect of this result is not the fact that there is variation in the intensity of the signal; it is the fact that only the Frontana cultivar demonstrates such a variation. This may suggest that the resistant cultivar is programmed with the right signalling tools to modulate an increase in transcription of the *wck-1* gene. Roblin, a much more susceptible cultivar, does not possess means to increase the transcription of the *wck-1* gene, known to be involved in stress responses.

Another surprising aspect of this part of the study, was that even after sequencing several bands from genomic DNA (data not shown), the only kinase to which homology was shown was the WCK-1 MAPK homologue in wheat. This may indicate that the PCR primer sets were more specific than intended or that the PCR conditions were too stringent to allow amplification of other MAPK

genes. When comparing the WCK-1-homologue to a wheat EST database comprising all the publicly available wheat ESTs, with their descriptions as well as being grouped into contigs, the WCK-1-homologue had 77-99% of homology to the consensus sequences of the contigs representing ~ 50 ESTs of kinases. This database was courtesy of ECORC and developed by Jiro Hattori. Also, some data mining of the database of ESTs produced at ECORC has been done. I have identified ~ 100 ESTs associated with different types of kinases in wheat (Appendix B). Experiments describing how these ESTs can be use to investigate signalling during infection will be described in the section Future Studies.

4.6 Conclusions

The western detection experiments showed variations in phosphorylation pattern, which differed within a cultivar as well as between cultivars. The differences between water-treated samples and *F. graminearum*-treated samples indicated some effects by *F. graminearum* on the wheat heads. Tyrosine residues and threonine residues were shown to vary in phosphorylation intensity and pattern at the 6-day sampling point. Also differences were observed between cultivars suggesting a differential response related to differing levels of resistance. Aside from the possible effects of *F. graminearum*, physiological phenomena occurring around 6 days post-anthesis may cause the changes in phosphorylation intensity. The different maturation periods of each cultivar may also have played a role. The in-tube kinase assays indicated the presence of an unknown component within the soluble protein extract would inhibit any potential

activity found in the extract. Therefore, this technical problem must be solved before any significant conclusions are drawn. Preliminary results from the in-gel kinase assays showed kinase activity constant throughout the samples at a molecular weight corresponding to a putative MAPK, suggesting that the activity was either not affected by the fungus or that the effects were not strong enough to be detected by the assay. Finally, preliminary results from the northern hybridization showed that mRNA levels of the *wck-1* gene varied only in the *F. graminearum*-treated 24 h Frontana sample. Therefore, we have observed a slight increase in *wck-1* transcript level coupled with a constant activity for all MAPKs.

4.7 Future studies

The problem identified in section 4.3 pertaining to the unknown inhibiting components in the protein extract must be addressed in order to be able to measure kinase activity in soluble protein extracts. This problem could be sidestepped by utilizing specific antibodies and immunoprecipitating the desired protein kinase(s), then conducting the kinase assay on the isolated kinase(s). The antibody used could range from highly specific (i.e. WIPK) to highly generalist (i.e. MAPK) with several rounds of immunoprecipitation to isolate many protein kinases. Thus, the isolated protein kinase would be relatively free of inhibitors/ phosphatases, allowing us to correctly measure kinase activity. Also, it would be important to determine the extent of the effects of SDS on protein kinase activity. This could be done by adding the active MAPK, erk2 to the

protein extraction buffer, without any soluble protein extract. If kinase activity is shown to be quenched by the extraction buffer, consequently extraction buffers without SDS would be examined as alternatives.

It is also important to determine the nature of the bands found in the western detection experiments. Are the bands, which migrate at the same molecular weight, part of the same protein? Therefore, are the ~ 40-45 kDa bands found in both anti-phospho-threonine and anti-phospho-tyrosine part of the same protein, or are they two distinct bands? Several steps could be taken in order to answer this question. Two-dimensional gels could be used to determine both the molecular weight and the iso-electric point of the proteins. The subsequent gels could then by blotted with the same antibodies used in this study. It is less likely that two distinct proteins will have the same molecular weight and share the same iso-electric point. In addition to this, would be to sequence bands. The resulting sequences would then be aligned with each other, to determine sequence similarities.

It is also important to determine if the bands that vary in phosphorylation in the presence of *F. graminearum* correlate with resistance. Firstly, this can be done by looking at other susceptible cultivars, as well as resistant cultivars, which share the same type I resistance as Frontana. If the results show some correlation between resistance levels and phosphorylation variations, then a more thorough approach can be taken. This could consist in crossing a resistant parent with a susceptible parent, who both show the expected phosphorylation variations. After a few generations of self-crossing the progenies, we would

develop several lines of homozygotes with varying resistance levels when assessed with fungal inoculations. The analysis would consist in measuring phosphorylation patterns and intensities of each line, following fungal inoculation and harvesting tissue after pre-determined time points. The different lines could possibly show significant variations in phosphorylation intensities and patterns, which could be correlated to resistance levels. Kinase activity could also be measured, and maybe showing some relationship with resistance levels.

Another problem mentioned above was the failure to isolate other kinases than WCK-1 homologues. This is being remedied by the production of subtractive hybridization libraries enriched in cDNAs expressed in wheat heads infected with *F. graminearum*, in comparison to wheat treated with water. After many rounds of sequencing of randomly picked clones and mining of the sequence database, several types of kinases were identified and the corresponding cDNAs amplified., thus producing a mini-bank of cDNAs corresponding only to different protein kinases. The remaining work will consist of fixing these cDNAs to a microscope slide and performing a low-density micro-array using RNA from wheat heads. Hybridizations will compare the level of expression of the selected genes between two RNA populations, ex: *F. graminearum*- versus water-treated samples. The analysis of the data should indicate if any of the kinases found in the subset on the slide are either up regulated or down regulated in presence of *F. graminearum*. The advantage of the micro-array technology is the possibility of observing several hundreds to several thousands of genes in response to a fungal pathogen. I have selected (*in silico*) so far, several hundred cDNAs

corresponding to kinases found in wheat. Certainly, there may be more in the wheat genome that have not be selected, yet this preliminary screening will give us some indications on future studies to conduct. If regulation is translational or post-translational, then the micro-array will not indicate any significant variations in cDNA levels. To study translational and post-translational regulation, classical biochemical methods can be used to answer several key questions, but with low-throughput. Thus, the need for high-throughput technology has brought about the development of peptide arrays. This technology allows for the identification of protein-protein interactions, the investigation of enzyme-substrate and enzyme-inhibitor interactions (Reimer *et al*, 2002).

The preliminary results of the northern hybridization must be expanded to include more time points with additional experiments. These experiments may include more spray and some point inoculation experiments.

Finally, the development of a cell culture line would facilitate the collection of data. This data acquisition would necessitate only a brief pathogen treatment in order to elicit a defence response. Many treatments could be observed in a shorter time period, therefore, answering many simple questions quickly. The limitation to this type of experiment is the lack of correlation to the physiology of the response. Hence, the response measured in the cell culture experiments would probably differ in intensity and response time, compared to experiments conducted *in planta*. Also, the three dimensional aspect of research done *in planta* produces significant cell-cell interactions, while the two-dimensional aspect of cell culture research can affect cell-cell interactions.

References

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

Alber, T. (1992) Structure of the leucine zipper. *Current Opinions in Genetics and Development*, **2**, 205-210.

Allwood, E., Davies, D., Gerrish, C., Ellis, B., Bolwell, G. (1999) Phosphorylation of phenylalanine ammonia-lyase: evidence for a novel protein kinase and identification of the phosphorylated residue. *FEBS Letters*, **457**, 47-52.

Atanasoff, D. (1920) Fusarium-blight (scab) of wheat and other cereals. *Journal of Agricultural Research*, **10**, 1-40

Atanassov, Z., Nakamura, C., Mori, N., Kaneda C. (1994) Mycotoxin production and pathogenicity of Fusarium species and wheat resistance to Fusarium head blight. *Canadian Journal of Botany*, **72**, 161-167.

Bai, G., Shaner, G., Ohm, H. (1993) Inheritance of resistance to Fusarium graminearum in eight wheat cultivars. *Phytopathology*, **83**, 1414.

Bai, G. and Shaner, G. (1994) Scab of wheat :Prospects of control. *Plant Disease*, **78**, 760-766.

Bechtel, D., Kaleikau L., Gaines, R., Seitz, L. (1985) The effects of Fusarium graminearum infection of wheat kernels. *Cereal Chemistry*, **62**, 191-197.

Becraft, P. (2002) Receptor kinase signalling in plant development. *Annual Reviews of Cell and Developmental Biology*, **18**, 163-192.

Bent, A. (1996) Plant disease resistance genes : Function meets structure. *The Plant Cell*, **8**, 1757-1771.

Berberich, T., Sano, H., Kusano, T. (1999) Involvement of a MAP kinase, ZmMPK5, in senescence and recovery from low-temperature stress in maize. *Molecular General Genetics*, **262**, 534-542.

Bisgrove, S., Simonich, M., Sattler, N., Innes, R. (1994) A disease resistance gene in Arabidopsis with specificity for two different pathogen avirulence genes. *Plant Cell*, **6**, 927-933.

Bogre, L., Ligterink, W., Herberle-Bors, E., Hirt, H. (1996) Mechanosensors in plants. *Nature*, **383**, 489-490.

Burgess L. (1981) General ecology of Fusaria. In : P.E. Nelson, T. A Tousson and R. J Cook (eds), *Fusarium* : Diseases, Biology, and Control. Pennsylvania State University Press, University Park. 225-235.

Cambier, V., Hance, T., de Hoffman, E. (2000) Variation of DIMBOA and related compounds content in relation to the age and plant organ in maize. *Phytochemistry*, **53**, 223-229.

Canagajarah, B., Khokhlatchev, A., Cobb, M., Goldsmith, E. (1997) Activation mechanism of the MAP kinase ERK2 by dual phosphorylation. *Cell*, **90**, 859-869.

Cardinale, F., Jonak, C., Ligterink, W., Niehaus, K., Boller, T., Hirt, H. (2000) Differential activation of four specific MAPK pathways by distinct elicitors. *The Journal of Biological Chemistry*, **275**, 36734-36740.

Chandok, M. and Sopory, S. (1998) ZmcPKC70, a protein kinase C-type

enzyme from maize. *The Journal of Biological Chemistry*, **273**, 19235-19242.

Chaudhuri, S., Seal, A., DasGupta, M. (1999) Autophosphorylation-dependent activation of a calcium-dependent protein kinase from groundnut. *Plant Physiology*, **120**, 859-866

Chico, J., Raices, M., Tellez-Inon, M., Ulloa, R. (2002) A calcium-dependent protein kinase is systemically induced upon wounding in tomato plants. *Plant Physiology*, **128**, 256-270.

Clear, R. and Patrick, S. (2002) Fusarium head blight in Canada, Mycology : Canadian Grain Commission, www.cgc.ca.

Cook, J. (1981) *Fusarium* diseases of wheat and other small grains in North America. In : P.E. Nelson, T. A Tousson and R. J Cook (eds), *Fusarium* : Diseases, Biology, and Control. Pennsylvania State University Press, University Park. 39-52

Cook, R., and Christen A. (1976) Growth of cereal root-rot fungi as affected by temperature-water potential interactions. *Phytopathology*, **66**, 193-197.

Cordero, M., Raventos, D., San Segundo, B. (1994) Differential expression and induction of chitinases and β-1,3-glucanases in response to fungal infection during germination of maize seeds. *Molecular Plant-Microbe Interactions*, **7**, 23-31.

Dangl, J. and Jones, J. (2001) Plant pathogens and integrated defense responses to infection. *Nature*, **411**, 826-833.

Dellaporta, S., Wood, J., Hicks, J. (1983) A plant DNA minipreparation: Version II. *Plant Molecular Biology Reporter*, **1**, 19-21.

Desjardins, A., and Hohn, T. (1997) Mycotoxins in Plant Pathogenesis. *Molecular Plant-Microbe Interactions*, **10**, 147-152.

Desjardins, A., Plattner, R., Proctor, H. (1996) Genetic and biochemical aspects of fumonisin production. *Advances in Experiments in Medical Biology*, **392**, 165-173.

Dill-Macky, R., and Jones, R. (1997) The effect of previous crops and tillage on Fusarium head blight of wheat. *Cereal Research Communication of the Fifth European Fusarium Seminar*, **25**, 711-712.

Droge-Laser, W., Kaiser, A., Lindsay, W., Halkier, B., Loake, G., Doerner, P., Dixon, R., Lamb, C. (1997) Rapid stimulation of a soybean protein-serine kinase that phosphorylates a novel bZIP DNA-binding protein, G/HBF-1, during the induction of early transcription-dependant defenses. *The EMBO Journal*, **16**, 726-738.

Droillard, M.-J., Thibivilliers, S., Cazalé, A.-C., Barbier-Brygoo, H. and Laurière, C. (2000) Protein kinases induced by osmotic stresses and elicitor molecules in tobacco cell suspensions: identification of two cross-roads MAP kinases and one osmoregulation-specific protein kinase. *FEBS Letters.*, **474**, 217-222.

Du, L. and Chen, Z. (2000) Identification of genes encoding receptor-like protein kinases as possible targets of pathogen- and salicylic acid-induced WRKY DNA-binding proteins in *Arabidopsis*. *The Plant Journal*, **24**, 837-847.

Ebel, J. and Mithofer, A. (1998) Early events in the elicitation of plant defense. *Planta*, **206**, 335-348.

Estruch, J., Kadwell, S., Merlin, E., Crossland, L. (1994) Cloning and characterization of a maize pollen-specific calcium-dependant calmodulin-independent protein kinase. *Proceedings of the National Academy of Science of the USA*, **91**, 8837-8841.

Feuillet, C., Schachermayr, G., Keller, B. (1997) Molecular cloning of a new receptor-like kinase gene encoded at the Lr10 disease resistance locus of wheat. *Plant Journal*, **11**, 45-52.

Fernando, W., Miller, J., Seaman, W., Seifert, K., Paulitz, T. (2000) Daily and seasonal dynamics of airborne spores of Fusarium graminearum and other Fusarium species sampled over wheat plots. *Canadian Journal of Botany*, **78**, 497-505.

Fielder, D., Collins, W., Blackwell, B., Bensimon, C., ApSimon, J. (1994) Isolation and characterization of 4-acetyl-benzoxazolin-2-one (4-ABOA), a new benzoxazolinone from *Zea mays*. *Tetrahedron Letters*, **35**, 521-524.

Flor, H. (1947) Host-parasite interactions in flax rust - Its genetics and other implications. *Phytopathology*, **45**, 680-685.

Francis R. and Burgess L. (1977) Characeristics of two populations of *Fusarium roseum 'graminearum'* in eastern Australia. *Transactions of the British Mycology Society*, **68**, 421-427.

Frye, C., Tang, D., Innes, R. (2001) Negative regulation of defense responses in plants by a conserved MAPKK kinase. *Proceedings of the National*

Academy of Sciences of the USA, **98**, 373-378.

Geahlen, R., Anostario, J., Low, P., Harrison, M. (1986) Detection of protein kinase activity in sodium dodecyl sulfate-polyacrylamide gels. *Analytical Biochemistry*, **153**, 151-158.

Gomez-Gomez, L., and Boller, T. (2000) FLS2: an LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in Arabidopsis. *Molecular Cell*, **5**, 1003-1011.

Gregory, P., Guthrie, E., Bunce, M. (1959) Experiments on splash dispersal of fungus spores. *Journal of General Microbiology*, **20**, 328-354.

Hanks S., Quinn, A., Hunter, T. (1988) The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. *Science*, **241**, 42-52.

Hardie, D. (1999) Plant protein serine/threonine kinases: Classification and functions. *Annual Reviews in Plant Physiology and Plant Molecular Biology*, **50**, 97-131.

Harmon, A., Putman-Evans, C., Cormier, M. (1987) A calcium-dependent but calmodulin-independent protein kinase from soybean. *Plant Physiology*, **83**, 830-837.

Harmon, A., Yoo, B., McCaffery, C. (1994) Pseudosubstrate inhibition of CDPK, a protein kinase with a calmodulin-like domain. *Biochemistry*, **33**, 7278-7287.

Harmon, A., Gribskov, M., Harper, J. (2000) CDPKs- a kinase for every Ca2+ signal? *Trends in Plant Sciences*, **5**, 154-159.

Hirt, H (2000) MAP kinases in plant signal transduction. *Results and Problems in Cell Differentiation*, **27**, 1-10.

Hohn, T., Desjardins, A., McCormick, S., Proctor, R. (1995) Biosynthesis of trichothecenes, genetic and molecular aspects. Molecular Approaches to Food Safety. M. Eklund, J.L. Richards and K. Mise, eds. Alaken, Fort Collins, 239-245.

Huang, J., Teyton, L., Harper, J. (1996) Activation of a Ca^{2+}-dependant protein kinase involves intramolecular binding of a calmodulin-like regulatory domain. *Biochemistry*, **35**, 13222-13230.

Huang, Y., Li, H., Gupta, R., Morris, P., Luan, S., Lieber, J. (2000) AtMPK4, an *Arabidopsis* homolog of mitogen-activated protein kinase, is activated *in vitro* by AtMEK1 through threonine phosphorylation. *Plant Physiology*, **122**, 1301-1310.

Hunter T. (1995) Protein kinases and phosphatases: the yin and yang of protein phosphorylation and signaling. *Cell*, **80**, 225-236.

Hutcheson, SW. (1998) Current concepts of active defence in plants. *Annual Reviews in Phytopathology*, **36**, 59-90.

Hutchcroft, J., Harrison, M., Geahlen, R. (1991) B lymphocyte activation is accompanied by phosphorylation of a 72-kDa protein-tyrosine kinase. *Journal of Biological Chemistry*, **266**, 14846-14849.

Ichimura, K., Mizoguchi, T., Yoshida, R., Yuasa, T., Shinozaki, K. (2000) Various abiotic stresses rapidly activate *Arabidopsis* MAP kinases AtMPK4 and AtMPK6. *Plant Journal*, **24**, 655-665.

Jonak, C., Heberle-Bors, E., Hirt, H. (1994) MAP kinases: universal multi-purpose signalling tools. *Plant Molecular Biology*, **24**, 407-416.

Jonak, C., Kiegerl, S., Ligterink, W., Barker, P., Huskisson, N., Hirt, H. (1996) Stress signalling in plants: a mitogen-activated protein kinase pathway is activated by cold and drought. *Proceedings of the National Academy of Sciences of the USA*, **93**, 11274-11279.

Jonak, C., Ligterink, W., Hirt, H. (1999) MAP kinases in plant signal transduction. *Cellular and Molecular Life Sciences*, **55**, 204-213.

Jonak, C., Beisteiner, D., Beyerly, J., Hirt, H. (2000) Wound-induced expression and activation of WIG, a novel glycogen synthase kinase 3. *Plant Cell*, **12**, 1467-1475.

Jones, A. and Dangl, J. (1996) Logjam at the styx: Programmed cell death in plants. *Trends in Plant Science*, **1**, 114-119.

Kajava, A. (1998) Strutural diversity of leucine-rich repeat proteins. *Journal of Molecular Biology*, **277**, 519-527.

Keigerl, S., Cardinale, F., Siligan, C., Gross, A., Baudouin, E., Liwosz, A., Eklof, S., Till, S., Bogre, L., Hirt, H., Meskiene, I. (2000) SIMKK, a mitogen-activated protein kinase (MAPK) kinase, is a specific activator of the salt stress-induced MAPK, SIMK. *Plant Cell*, 12, 2247-2258.

Keen, N. (1990) Gene-for-gene complementarity in plant-pathogen interactions. *Annual Reviews in Genetics*, **24**, 447-463.

Keyse, S. (2000) Protein phosphatases and the regulation of mitogen-activated protein kinase signalling. *Current Opinion in Cellular Biology*, **12**, 186-

192.

Kobe, B. and Deisenhofer, J. (1994) The leucine-rich repeat: A versatile binding motif. *Trends in Biochemical Sciences*, **19**, 415-421.

Kooman-Gersmann, M., Honee, G., Bonnema, G., De Wit, P. (1996) A high-affinity binding site for the AVR9 peptide elicitor of *Cladosporium fulvum* is present on plasma membranes of tomato and other solanaceous plants. *Plant Cell*, **8**, 929-938.

Kotsonis, F., Smalley, E., Ellison, R., Gale, C. (1975) Feed refusal factors in pure cultures in Fusarium roseum 'graminearum'. *Applied Microbiology*, **30**, 362-368.

Laemmli, U. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680-685.

Lam, E., Kato, N., Lawton, M. (2001) Programmed cell death, mitochondria and the plant hypersensitive response. *Nature*, 411, 848-853.

Levine, A., Tenhaken, R., Dixon, R., Lamb, C. (1994) H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. *Cell*, **79**, 583-593.

Li, Z., Wurtzel, E. (1998) The Itk gene family encodes novel receptor-like kinases with temporal expression in developing maize endosperm. Plant Molecular Biology, **37**, 749-761.

Ligterink, W., Kroj, T., zur Nieden, U., Hirt, H., Scheel, D. (1997) Receptor-mediated activation of a MAP kinase in pathogen defense of plants. *Science*, **276**, 2054-2057.

Ligterink, W. (2000) MAP kinases in plant signal transduction: How many and what for ? *Results and Problems in Cell Differentiation*, **27**, 11-27.

Liu, Y., Zhang, S., Klessig, D. (2000) Molecular cloning and characterization of a tobacco MAP kinase kinase that interacts with SIPK. *Molecular Plant-Microbe Interactions*, **13**, 118-124.

Lodge, J., Kaniewski, W., Tumer, N. (1993) Broad spectrum virus resistance in transgenic plants expressing pokeweed antiviral protein. *Proceedings of the National Academy of Science of the U.S.A*, **90**, 7089-7093.

Martin, G., Brommonschenkel, S., Chunwongse, J., Frary, A., Ganal, M., Spivez, R., Wu, T., Earle, E., Tanksley, S. (1993) Map-based cloning of a protein kinase gene conferring disease resistance in tomato. *Science*, **262**, 1432-1436.

Meeley, R., Johal GS, Briggs SP, Walton JD. (1992) A biochemical phenotype for a disease resistance gene of maize. *The Plant Cell*, **4**, 71-77.

Meskiene, I., Baudoin, E., Schweighofer, A., Liwosz, A., Jonak, C., Rodriguez, P., Jelinek, H., Hirt, H. (2003) The stress-induced protein phosphatase 2C is a negative regulator of a mitogen-activated protein kinase. *Journal of Biological Chemistry*, **23**, 18945-18952.

Mesterhazy, A. (1995) Types and components of resistance to Fusarium head blight of wheat. *Plant Breeding*, **114**, 377-386.

Mesterhazy, A. (1997) Methodology of resistance testing and breeding against Fusarium head blight in wheat and results of the selection. *Fifth European Fusarium Seminar*, 631-637.

Miedaner, T., Borchardt, D., Geiger, H. (1993) Genetic analysis of inbred lines and their crosses for resistance to headblight (Fusarium culmorum, F. graminearum) in winter rye. *Euphytica*, **65**, 123-133.

Miedaner, T. (1997) Breeding wheat and rye for resistance to Fusarium diseases. *Plant Breeding*, **116**, 201-220.

Mikoljczyk, M., Awotunde, O., Muszynska, G., Klessig, D., Dobrowolska, G. (2000) Osmotic stress induces rapid activation of a salicylic acid-induced protein kinase and a homolog of protein kinase ASK1 in tobacco cells. *The Plant Cell*, **12**, 165-178.

Miller, J. and Arnison P. (1986) Degradation of deoxynivalenol by suspension cultures of the Fusarium head blight resistant wheat cultivar Frontana. *Canadian Journal of Plant Pathology*, **8**, 147-150.

Miller, J., Fielder, D., Dowd, P., Norton, R., Collins, W. (1996) Isolation fo 4-acetyl-benzoxazolin-2-one (4-ABOA) and diferuloylputrescine from an extract of Gibberella ear rot-resistant corn that blocks mycotoxin biosynthesis, and the insect toxicity of 4-ABOA and related compounds. *Biochemical Systematics and Ecology*, **24**, 647-658.

Mizoguchi, T., Irie, K., Hirayama, T., Hayashida, N., Yamaguchi-Shinozaki, K., Matsumoto, K., Shinozaki, K. (1996) A gene encoding a mitogen-activated protein kinase kinase kinase is induced simultaneously with genes for a mitogen-activated protein kinase and S6 ribosomal protein kinase by touch, cold and water stress in Arabidopsis thaliana. *Proceedings of the National Academy of Sciences of the USA*, **93**, 765-

769.

Mizoguchi, T., Ichimura, K., Irie, K., Morris P., Giraudat, J., Matsumoto, K., Shinozaki, K. (1998) Identification of a possible MAP kinase cascade in Arabidopsis thaliana based on pairwise yeast two-hybrid analysis and functional complementation tests of yeast mutants. *FEBS Letters*, **437**, 56-60.

Morris, P., Guerrier, D., Leung, J., Giraudat, J. (1997) Cloning and characterization of *MEK1*, an *Arabidopsis* gene encoding a homologue of MAP kinase kinase. *Plant Molecular Biology*, **35**, 1057-1064..

Murillo, I., Jaeck, E., Cordero, J., San Segundo, B. (2001) Transcriptional activation of a maize calcium-dependant protein kinase gene in response to fungal elicitors and infection. *Plant Molecular Biology*, **45**, 145-158.

Newton, A. (1995) Protein kinase C. Seeing two domains. *Current Biology*, **5**, 973-976

Okubara, P., Blechl, A., McCormick, S., Alexander, N., Dill-Macky, R., Hohn, T. (2002) Engineering deoxynivalenol metabolism in wheat through the expression of a fungal trichothecene acetyltransferase gene. *Theory of Applied Genetics*, 106, 74-83.

Parker, J. and Coleman, M. (1997) Molecular intimacy between proteins specifying plant-pathogen recognition. *Trends in Biological Sciences*, **22**, 291-295.

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

Perez, F. and Niemeyer, H. (1989) Reaction of DIMBOA, a resistance factor from cereals, with papain. *Phytochemistry*, **6**, 1597-1600.

Perkowski, J., Chelkowski, J., Wakulinski, W. (1990) Mycotoxins in cereal grain. Part 13. Deoxynivalenol and 3-acetyl-deoxynivalenol in wheat kernels and chaff with head fusariosis symptoms. *Die Nahrung*, **34**, 325-328.

Petersen, M., Brodersen, P., Naeste, H., Andreasson, E., Lindhart, U., Johansen, B., Nielsen, H., Lacy, M., Austin, M., Parker, J., Sharma, S., Klessig, D., Martienssen, R., Mattsson, O., Jensen, A., Mundy, J. (2000) Arabidopsis MAP kinase 4 negatively regulates systemic acquired resistance. *Cell*, **103**, 1111-1120.

Pritsch, C., Muehlbauer, G., Bushnell, W., Somers, D., Vance, C. (2000) Fungal development and induction of defense response genes during early infection of wheat spikes by Fusarium graminearum. *Molecular Plant-Microbe Interactions*, **13**, 159-169.

Proctor, R., Hohn, T., McCormick, S. (1995) Reduced virulence of Gibberella zeae cuased by disruption of a trichothecene toxin biosynthesis gene. *Molecular Plant-Microbe Interactions*, **8**, 593-601.

Pugh G., Johann, H., Dickson, J. (1933) Factors affecting infection of wheat heads by Gibberella saubinetii. *Journal of Agricultural Research*, **46**, 771-797.

Reid, L., Zhu, X., Savard, M., Sinha, R., Vigier, B. (2000) Pre-harvest accumulation of deoxynivalenol in sweet corn ear inoculated with

Fusarium graminearum. *Food additives and contaminants*, **17**, 689-701.

Reimer, U., Reineke, U., Schneider-Mergener, J. (2002) Peptide arrays: from macro to micro. *Current opinions in Biotechnology*, **13**, 315-320.

Rintamaki, E., Salonen, M., Suoranta, U., Carlberg, I.,, Andersson, B., Aro, E. (1997) Phosphorylation of light-harvesting complex II and photosystem II core proteins shows different irradiance-dependant regulation *in vivo*. *The Journal of Biological Chemistry*, **272**, 30476-30482.

Romeis, T., Piedras, P., Zhang, S., Klessig, D., Hirt, H., Jones, J. (1999) Rapid *Avr9* and *Cf9* dependant activation of MAP kinases in tobacco cell cultures and leaves: convergence of resistance gene, elicitor, wound and salicylic acid responses. *Plant Cell*, **11**, 273-287.

Romeis, T., Piedras, P., Jones, J (2000) Resistance gene-dependent activation of a calcium-dependent protein kinase in the plant defense response. *Plant Cell*, **12**, 803-816

Romeis, T., Ludwig, A., Martin, R., Jones, J. (2001) Calcium-dependant protein kinase play an essential role in a plant defense response. *The EMBO Journal*, **20**, 5556-5567.

Rossi, M., Goggin, F., Milligan, S., Kaloshian, I., Ullman, D., Williamson, V. (1998) The nematode resistance gene *Mi* of tomato confers resistance against the potato aphid. *Proceedings of the National Academy of Science of the USA*, **95**, 9750-9754.

Rousseaux, C. (1988) Trichothecene Mycotoxins : Real or Imaginary Toxins? *Comments in Toxicology*, **2**, 37-49.

Ruis, H. and Schuller, C. (1995) Stress signalling in yeast. *Bioessays*, **17**, 959-965.

Saijo, Y., Hata, S., Kyozuka, J., Shimamoto, K., Izui, K. (2000) Over-expression of a single Ca2+-dependant protein kinase confers both cold and salt/drought tolerance on rice plants. *The Plant Journal*, **23**, 319-327.

Saraste, M., Sibbald, P., Wittinghofer, A. (1990) The P-loop-a common motif in ATP- and GTP- binding proteins. *Trends in Biotechnology*, **15**, 430-435.

Schroeder, H. and Christensen, J. (1963) Factors affecting resistance of wheat to scab caused by Gibberella zeae. *Phytopathology*, **53**, 831-838.

Seo, S., Sano, H., Ohashi, Y. (1999) Jasmonate-based wound signal transduction requires activation of WIPK, a tobacco mitogen-activated protein kinase. *Plant Cell*, **11**, 289-298.

Silva, N., Christie, L., Mazzurco, M., Goring, D. (2001) Characterization of a novel *Brassica napus* kinase, *BNK1*. *Plant Science*, **160**, 611-620.

Snijders, C. (1990) Fusarium head blight and mycotoxin contamination of wheat, a review. *Netherlands Journal of Plant Pathology*, **96**, 187-198.

Snijders, C., and Perkowski, J. (1990) Effects of headblight caused by Fusarium culmorum on toxin content and weight of wheat kernels. *Phytopathology*, **80**, 566-570.

Song F, Goodman RM. (2002) OsBIMK1, a rice MAP kinase gene involved in disease resistance responses. *Planta*, 215, 997-1005.

Song, W., Wang, G., Chen, L ., Kim, H., Pi, L., Holsten, T., Gardner, J., Wang, B., Zhai, W., Zhu, L ., Fauquet, C., Ronald, P. (1995) A receptor kinase-

like protein encoded by the rice disease resistance gene, *Xa21*. *Science*, **270**, 1804-1806.

Stack, R. (1999) Return of an old problem : Fusarium head blight of small grains. APSnet : *Plant Pathology on-line*, May 1-31 Feature, 1-10.

Staskawicz, B. (2001) Genetics of plant-pathogen interactions specifying plant disease resistance. *Plant Physiology*, **125**, 73-76.

Strange, R. and Smith, H. (1971) A fungal growth stimulant in anthers which predisposes wheat to attack by Fusarium graminearum. *Physiology of Plant Pathology*, **1**, 141-150.

Strange, R., Deramo, A., Smith, H. (1978) Virulence enhancement of Fusarium graminearum by choline and betaine and of *Botrytis cinerea* (grey mold) by other constituents of wheat germ. *Transactions of the British Mycological Society*, **70**, 201-207.

Strausbaugh, C. and Maloy, O. (1986) Fusarium scab of irrigated wheat in Central Washington. *Plant Disease*, **70**, 1104-1106.

Subramaniam, R., Després, C.., Brisson, N. (1997) A functional homolog of a mammalian protein kinase C participates in the elicitor-induced defense response in potato. *Plant Cell*, **9**, 653-664.

Sung, J., and Cook R. (1980) Effects of water potential on reproduction and spore germination of Fusarium graminearum , F. culmorum and F. avenaceum. *Phytopathology*, **71**, 499-504.

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

209.

Takezawa, D. (1999) Elicitor- and A23187-induced expression of WCK-1, a gene encoding mitogen-activated protein kinase in wheat. *Plant Molecular Biology*, **40**, 921-933.

Taraporewala, Z., and Culver, J. (1996) Identification of an elicitor active site within the three-dimensional structure of the tobacco mosaic tobamovirus coat protein. *Plant Cell*, **8**, 169-178.

Tshcanz, A., Horst, R., Nelson, P. (1976) The effects of environment on sexual reproduction of Gibberella zeae. *Mycologia*, **68**, 327-340.

Ueno, Y. (1977) Trichothecenes : Overview Address. Mycotoxins in human and animal health. Pathotox Publishers. 189-207.

Usami, S., Banno, H., Ito, Y., Nishihama, R., Machida, Y. (1995) Cutting activates a 46-kilodalton protein kinase in plants. *Proceedings of the National Academy of Sciences of the USA*, **92**, 8660-8664.

van Kan, J., van den Ackerveken, G., De Wit, P. (1991) Cloning and characterization of cDNA of avirulence gene *avr9* of the fungal pathogen Cladosporium fulvum, causal agent of tomato leaf mold. *Molecular Plant-Microbe Interaction*, **4**, 52-59.

Vesonder, R.F. and Hesseltine, C.W. (1981) Metabolites of *Fusarium*. In : P.E. Nelson, T. A Tousson and R. J Cook (eds), *Fusarium* : Diseases, Biology, and Control. Pennsylvania State University Press, University Park. 350-364.

Wang, Y., and Miller, J. (1988) Effects of Fusarium graminearum metabolites on

wheat tissue in relation to Fusarium head blight resistance. *Journal of Phytopathology*, **122**, 118-125.

Wang, X., Zafian, P., Choudhary, M., Lawton, M. (1996) The PR5K receptor protein kinase from *Arabidopsis thaliana* is structurally related to a family of plant defense proteins. *Proceedings of the National Academy of Science of the USA*, **93**, 2598-2602.

Wearing, A., and Burgess, L. (1977) Distribution of Fusarium roseum 'Graminearum' Group I and its mode of survival in eastern Australia wehat belt soils. *Transactions of the British Mycology Society*, **69**, 429-442.

Weretilnyk, E., Alexander, K., Drebenstedt, M., Snider, J., Summers, P., Moffat. B. (2001) Maintaining methylation activities during salt stress: the involvement of adenosine kinase. *Plant Physiology*, **125**, 856-865.

Whitehurst AW, Wilsbacher JL, You Y, Luby-Phelps K, Moore MS, Cobb MH. (2002) ERK2 enters the nucleus by a carrier-independent mechanism. *Proceedings of the National Academy of Science of the USA*, **99**, 7496-7501.

Windels, C. and Kommedahl, T. (1974) Population differences in indigenous Fusarium species by corn culture of prairie soil. *American Journal of Botany*, **61**, 141-145.

Windels, C., Windels, M., Kommedahl, T. (1976) Association of Fusarium species with picnic beetles on corn ears. *Phytopathology*, **66**, 328-331.

Xiong, L., Yang, Y. (2003) Disease resistance and abiotic stress tolerance in rice are inversely modulated by an abscisic acid-inducible mitogen-

activated protein kinase. *The Plant Cell*, **15**, 745-759.

Xu, Q., Fu, H., Gupta, R., Luan, S. (1998) Molecular characterization of a tyrosine-specific protein phosphatase encoded by a stress-responsive gene in *Arabidopsis*. *The Plant Cell*, **10**, 849-857.

Yang, K., Lui, Y., Zhang, S. (2001) Activation of a mitogen-activated protein kinase pathway is involved in disease resistance in tobacco. *Proceedings of the National Academy of Science of the USA*, **98**, 741-746.

Yoon, G., Cho, S., Ha, H., Lui, J., Lee, H. (1999) Characterization of NtCDPK1, a calcium-dependant protein kinase gene in *Nicotiana tabacum*, and the activity of its encoded protein. *Plant Molecular Biology*, **39**, 991-1001.

Zhang, S. and Klessig, D. (1997) Salicylic acid activates a 48-kD MAP kinase in tobacco. *Plant Cell*, **9**, 809-824.

Zhang, S. and Klessig, D. (1998) Resistance gene *N*-mediated *de novo* synthesis and activation of a tobacco mitogen-activated protein kinase by tobacco mosaic virus infection. *Proceedings of the National Academy of Sciences of the USA*, **95**, 7433-7438.

Zhang, S. and Lui, Y. (2001) Activation of salicylic acid-induce protein kinase, a mitogen-activated protein kinase, induces multiple defense responses in tobacco. *Plant Cell*, **13**, 1877-1889.

Zhou, J., Loh, Y., Bressan, R., Martin, G. (1995) The tomato gene *Pti1* encodes a serine/threonine kinase that is phosphorylated by *Pto* and is involved in the hypersensitive response. *Cell*, **83**, 925-935.

Appendix A :

Composition of Buffers :

40% (w/v) Acrylamide
100g of acrylamide
Dissolve in 250ml of ddH_2O
Filter sterilize.
Store at 4°C in the dark for up to 4 weeks.

1 % Ammonium persulfate
Dissolve 0.1g in 10ml of ddH_2O.
Use immediately.

2% (w/v) N,N-methylene-bisacrylamide
2g of N,N-methylene-bisacrylamide
Dissolve into 100ml of ddH_2O
Filter sterilize.
Store at 4°C in the dark for up to 4 weeks.

Coomassie blue G-250 staining solution
200ml of acetic acid
1800ml of ddH_2O
0.5g of Coomassie blue G-250
Mix 1hr and filter (Whatman no.1 paper)
Store at room temperature

50X Denhardt's Solution
5 g of Ficoll
5 g of polyvinylpyrrolidone
5 g of bovine serum albumin
Adjust to 500 ml with ddH_2O
Filter sterilize
Store at -20C

1M DTT
0.17g of DTT
Dissolve in 1ml of ddH_2O. Use immediately.

0.5M EDTA
186.1g of disodium ethylenediaminetetraacetic acid
Dissolve in 800ml of ddH_2O
Adjust pH to 8.0
Sterilize by autoclaving
Store at 4°C

0.17M KH_2PO_4, 0.72M K_2HPO_4 solution
2.31g of KH_2PO_4
12.54 of K_2HPO_4
Dissolve in 100ml of ddH_2O
Sterilize by autoclaving
Store at 4°C

10X Kinase Activity buffer
200mM Pipes, pH 7.0
100mM $MgCl_2$
20mM $MnCl_2$
100mg/ml of aprotinin
Use fresh

LB Agar media
5g of bacto-tryptone
2.5g of bacto-yeast extract
5g of NaCl
Dissolve in 500ml of ddH_2O
Add 7.5g of agar
Mix and sterilize by autoclaving

1M $MgCl_2$
203.3g of $MgCl_2$
Adjust to 1L with ddH_2O
Sterilize by autoclaving
Store at 4°C

1M $MnCl_2$
125.8g of $MnCl_2$
Adjust to 1L with ddH_2O
Store at 4°C

10X MOPS
0.2M MOPS
50mM sodium acetate
10mM EDTA, pH 8.0
Adjust pH to 7.0
Sterilize by autoclaving

100mM PMSF
0.015g of PMSF
Dissolve in 1ml of isopropanol.
Use immediately.

Protein Electrophoresis buffer
56.44g of glycine
12g of Tris base
4g of SDS
Dissolve to 4L with ddH_2O
Store at 4°C

Protein Loading buffer
7ml of 1M Tris-HCl pH 6.8
3ml of glycerol
1g of SDS
0.93g of DTT
1.2mg of bromophenol blue
Adjust to 10ml with ddH2O.
Prepare 0.5mL aliquot s.
Store at -70°C

Protein Transfer buffer
6g of Tris base
28.8g of glycine
Dissolve in 1600ml of ddH_2O
Add 400ml of methanol
Store at 4°C

QCEB (DNA Extraction buffer)
100 mM Tris-HCl, pH. 8.0
50mM EDTA
500mM NaCl
Use fresh

20 X SSC
175.3 g of NaCl
88.2 g of sodium citrate
Adjust to pH. 7.0
Adjust to 1L with ddH_2O
Sterilize by autoclaving
Store at room temperature

10% SDS
100g of electrophoresis-grade SDS
Add 900 ml of ddH_2O
Heat to 68°C dissolve
Adjust to 1L with ddH_2O
Store at room temperature

3X SDS sample buffer
187.5mM Tris-HCl, pH 7.0
6% (w/v) SDS
0.03% phenol red
Store at room temperature

SOC medium
20g of bacto-tryptone
5g of bacto-yeast extract
0.5g of NaCl
Dissolve in 950ml of ddH_2O
Add 10ml of 250mM KCl
Adjust pH to 7.0
Adjust to 1L with ddH_2O
Sterilize by autoclaving.
Cool and add 20ml of 1M glucose
Store at 4°C

3M Sodium acetate
408.1g of sodium acetate
Add 800ml of ddH_2O
Adjust pH to 7.0
Adjust to 1L with ddH_2O
Sterilize by autoclaving
Store at 4°C

Terrific Broth (TB)
12g of bacto-tryptone
24g of bacto-yeast extract
4ml of glycerol
Dissolve in 900ml of ddH_2O
Sterilize by autoclaving
Cool and then add 100ml of 0.17M KH_2PO_4, 0.72M K_2HPO_4 solution
Store at 4°C

10X TBS
100ml of Tris-HCl, pH 8.0
87.66g of NaCl
Adjust to 1L with ddH_2O
Store at room temperature

1X TBS + Tween 20 (0.1%)
Add 25ml of 10X TBS
Adjust to 250ml with ddH_2O
Add 250ul of Tween-20
Store at room temperature

TE buffer
10mM of Tris-HCl (pH 8.0)
1mM EDTA (pH 8.0)
Store at 4°C

50X Tris-acetate/EDTA(TAE)
242 g Tris base
57.1 ml of glacial acetic acid
100 ml of 0.5M EDTA, pH 8.0
Adjust to 1L with ddH_2O
Store at room temperature

1M Tris-HCl
121.1g of Tris base
Add 800ml of ddH_2O
Adjust to required pH with
concentrated HCl
Adjust to 1L with ddH_2O
Sterilize by autoclaving
Store at room temperature

X-Gal
Stock solution of 20 mg/ml
Store at -20°C in dark.

Appendix B

List of sequenced clones picked to eventually be fixed on micro-array

Ta01_01e09	Singleton	calcium-dependent protein kinase 19 - Arabidopsis thaliana (fragment)
Ta01_14b02	ecta48	putative protein kinase [Arabidopsis thaliana]
Ta01_09c07	ecta1040	probable serine/threonine-specific protein kinase (EC 2.7.1.-) A_IG002N01.22 -
Ta01_12h06	Singleton	probable serine/threonine/tyrosine-specific protein kinase (EC 2.7.1.-) T9I4.1 -
Ta01_16c06	Singleton	putative glycerol kinase [Arabidopsis thaliana]
Ta03_04g12	Singleton	hypothetical protein T21L14.21 - Arabidopsis thaliana gi|2702277|gb|AAB91980.1|
Ta01_08f12	ecta650	hypothetical protein F4I18.27 - Arabidopsis thaliana
Ta01_13b05	ecta1262	receptor-protein kinase-like protein - Arabidopsis thaliana gi|6572076|emb|CAB63019.1|
Ta01_02h07	Singleton	Strong similarity to gb|AF096285 serine-threonine kinase receptor-associated protein
Ta01_08f07	Singleton	receptor-like protein kinase homolog [Arabidopsis thaliana]
Ta01_12b01	Singleton	Similar to putative NAK-like Ser/Thr protein kinase. (AF001308)
Ta03_02a10	Singleton	S-receptor kinase (EC 2.7.1.-) Ark1 precursor - Arabidopsis thaliana gi|2129704|pir|
Ta03_08e12	Singleton	receptor protein kinase-like protein - Arabidopsis thaliana gi|7960721|emb|CAB92043.1|
Ta01_14d02	Singleton	SbRLK6 Sorghum bicolor cv. TX430 similar to protein kinase, highest similarity to receptor-lil
Ta01_14b06	Singleton	receptor protein kinase-like protein [Arabidopsis thaliana]
Ta03_11b04	ecta1192	leucine-rich repeat transmembrane protein kinase 2 - maize
Ta01_14d02	Singleton	SbRLK6 Sorghum bicolor cv. similar to protein kinase, highest similarity to receptor-like
Ta01_20c10	Singleton	galactose kinase [Arabidopsis thaliana]
Ta01_11e11	ecta193	PHOSPHORIBULOKINASE PRECURSOR (PHOSPHOPENTOKINASE) (PRKASE) (PRK)
Ta04_02a05	Singleton	protein kinase CK2 regulatory subunit CK2B3 [Zea mays]
Ta03_02d05	ecta447	PHOSPHOGLYCERATE KINASE, CHLOROPLAST PRECURSOR
Ta01_05g02	Singleton	PHOSPHOGLYCERATE KINASE, CYTOSOLIC
Ta03_09a03	Singleton	pyruvate kinase [Arabidopsis thaliana]
Ta03_11b06	Singleton	protein kinase homolog F14M4.11 - Arabidopsis thaliana gi|3522961|gb|AAC34243.1|
Ta03_08g06	Singleton	SHAGGY RELATED PROTEIN KINASE ASK-ALPHA
Ta03_11a11	Singleton	AC051634_22 Ste20-related protein kinase [Oryza sativa]
Ta01_05d08	ecta168	Similar to protein kinase C substrate (P14314) [Oryza sativa]
Ta01_09a03	ecta1039	TUBULIN ALPHA CHAIN
Ta01_06f04	ecta939	Barley beta-1,3-glucanase (GLU2) gene, exons 1-2

Ta01_05g07	ecta778	Porteresia coarctata histone H3 mRNA, complete cds
Ta01_06h08	Singleton	EREBP-like protein [Oryza sativa]
Ta01_09f01	Singleton	60S ribosomal protein L2 [Arabidopsis thaliana]
Ta02_03e07	ecta1094	Oryza sativa genomic DNA, chromosome 1, PAC clone:P0537A05, complete sequence
Ta04_02d01	Singleton	stress-induced protein sti1-like protein - Arabidopsis thaliana gi|5281051|emb|CAB45987.1|
Ta03_11g02	ecta103	Wheat cold-stressed seedling cDNA library Triticum aestivum cDNA clone WHE0368_E10_I
Ta01_06h07	Singleton	AAF68391.1 hypersensitive-induced response protein [Zea mays]
Ta04_03d04	Singleton	AAF68391.1 hypersensitive-induced response protein [Zea mays]
Ta01_06a01	ecta918	actin 1 [imported] - small naked oat gi|7211789|gb|AAF40438.1|
Ta01_07f03	ecta918	ACTIN 97 >gi|100417|pir||S20098 actin - potato
Ta01_18f04	Singleton	diacylglycerol kinase (ATDGK1) [Arabidopsis thaliana]
Ta01_17d05	ecta231	superoxide dismutase (EC 1.15.1.1) (Cu-Zn) 2, chloroplast - wheat gi|1572627|gb|AAB67991
Ta01_09g10	ecta333	SUPEROXIDE DISMUTASE [CU-ZN] 4A
Ta01_05h09	Singleton	Null entry (no hit, or not blasted yet)
Ta01_05d04	Untested	Null entry (no hit, or not blasted yet)
Ta01_04b06	Singleton	Null entry (no hit, or not blasted yet)
Ta01_12a07	ecta389	calnexin - maize (fragment) gi|1181331|emb|CAA54678.1| (X77569) calnexin [Zea mays]
Ta03_04c02	ecta117	CALNEXIN HOMOLOG PRECURSOR
Ta02_02d03	Singleton	chitinase (EC 3.2.1.14) - wheat
Ta03_04a01	ecta1330	class III chitinase [Oryza sativa subsp. japonica]
Ta03_01a03	ecta971	transcription factor [Oryza sativa]
Ta01_04e09	ecta964	ELONGATION FACTOR 1-ALPHA (EF-1-ALPHA)
Ta01_04h11	Singleton	transcription factor CRC [Arabidopsis thaliana]
Ta01_09h01	ecta966	Wheat pre-anthesis spike cDNA library Triticum aestivum cDNA clone WHE2320_B05_D10
Ta01_07h02	Singleton	Wheat pre-anthesis spike cDNA library Triticum aestivum cDNA clone WHE0959_D07_G13
Ta02_04d04	Singleton	Oryza sativa thymidine kinase (TK) mRNA, complete cds
Ta03_02d07	Singleton	PROTEIN KINASE G11A
Ta01_08g08	Singleton	adenosine kinase [Zea mays]
Ta01_20c10	Singleton	galactose kinase [Arabidopsis thaliana]
Ta01_08d11	Singleton	GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE, CYTOSOLIC
Ta04_03a12	Singleton	NBS-LRR type resistance protein - rice (fragment) gi|2792238|gb|AAB96994.1|
Ta03_08h11	Singleton	HEXOKINASE 1
Ta03_09f12	Singleton	HEXOKINASE 1

Appendix C

Control experiment where a typical western hybridization using the anti-phospho-threonine and the secondary antibody is shown in A. In B, a western hybridization is conducted on the same protein extracts using only the secondary antibody, in order to determine the non-specific binding activity of this antibody.