Wheat Mitogen-Activated Protein Kinase Pathways in Plant Defense Response

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Abstract

Plants are constantly exposed to a variety of abiotic stresses such as high or low temperatures, drought, or high salinity, and biotic stresses such as pathogen infection or insect attack. Plants respond to these stresses through the activation and coordination of various pathways. This activation and coordination requires the phosphorylation of proteins. To date, many kinases, phosphatases and other molecules involved in phosphorylation pathways have been characterized. Mitogen activated protein kinase (MAPK) pathway is one of the key phosphorylation pathways. This pathway consist of at least three core enzymes a MAPK, activated by a MAPK kinase, which is in turn activated by a MAPK kinase kinase. Also, programmed cell death is recognized as an essential physiological and genetic process during plant development and in response to biotic and abiotic stresses. In this study, we have identified wheat FLR and FLRS genes, which are MAPKK kinase and MAP kinase respectively, and FLRS has been further identified as an ERK-type MAPK. RT-PCR has revealed that FLR and FLRS transcript levels are not changed in salicylic acid (SA) and fumonisin B1 (FB1) treatments, suggesting that FLR and FLRS may not be involved in SA-mediated pathway, and they may not play a role in wheat response to FB1 at transcriptional level. However, western blotting analysis using anti-^{P}TEY^{P} antibody has indicated that ERK-type MAPKs (including FLRS) in wheat FHB resistant cultivar Frontana could be involved in the SA signaling pathway, and ERK-type MAPKs play a role in wheat defense against FB1 toxin at post-translational level. Further RT-PCR has indicated that PR1.2 may not be downstream of ERK-type MAPK pathway in wheat, and PR1.2 was unresponsive to SA treatment. Northern blotting analysis has shown that FLR and FLRS are transcriptionally up-regulated upon incompatible leaf rust challenge and *Fusarium graminearum* challenge, indicating that these two genes play a role in plant defense response to leaf rust and *Fusarium graminearum* challenges. Therefore, potentially it is possible that we could manipulate FLR and FLRS for plant disease resistance. Furthermore, SA and FB1 treatments induced cell death in wheat, which appeared to be necrosis and associated with concurrent accumulation of ROS. Further study will help delineate the pathways and function of FLR and FLRS genes in plant growth and development and in cell death regulation.

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ABBREVIATIONS

BLAST: Basic Local Alignment Search Tool

cDNA: complementary DNA

DEPC: diethylpyrocarbonate

ECL: enhanced chemiluminescence

EDTA: ethylenediamine tetraacetic acid

EtBr: ethidium bromide

FB1: fumonisin B1

kb: kilobase

kDa: kilo Dalton

PCD: programmed cell death

MAPK: Mitogen-Activated Protein Kinase

mRNA: messenger RNA

NCBI: National Center for Biotechnology Information

PAGE: polyacrylamide gel electrophoresis

PCR: polymerase chain reaction

RT-PCR: reverse-transcriptase polymerase chain reaction

PVDF: polyvinylidine fluoride

SA: salicylic acid

SDS: sodium dodecyl sulphate

TEMED: N,N,N',N'-tetramethylethylenediamine

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Chapter I

General Introduction

1.1 Protein phosphorylation

Plants are constantly exposed to a variety of abiotic stresses such as high or low temperatures, drought, or high salinity, and biotic stresses such as pathogen infection or insect attack. To survive these challenges, plants have developed elaborate mechanisms to detect external signals and manifest adaptive responses with proper physiological and morphological changes (Bohnert *et al.*, 1995).

Protein phosphorylation plays an important role in regulating and coordinating pathways in plants that sense and respond to environmental stresses and pathogen attacks, and it is one of the best understood processes involved in the regulation of protein functions in plant cells. The phosphorylation of a protein can change its behaviour in almost every conceivable way, including its intrinsic biological activity, half life, subcellular location and docking with other proteins or DNAs (Xing *et al.,* 2002). Therefore, phosphorylation is essential for the integration of signals within the cell. The collective effect of the complex phosphorylation machinery can determine the final effectiveness of defense mechanisms. Within the *Arabidopsis* genome, there are about 1000 protein kinase genes and about 200 phosphatase genes (The *Arabidopsis* genome initiative, 2000). Many kinases, phosphatases, and other molecules that are involved in phosphorylation pathways have been studied in the past a few decades (Zhang and Klessig, 2001; Xing *et al.*, 2002).

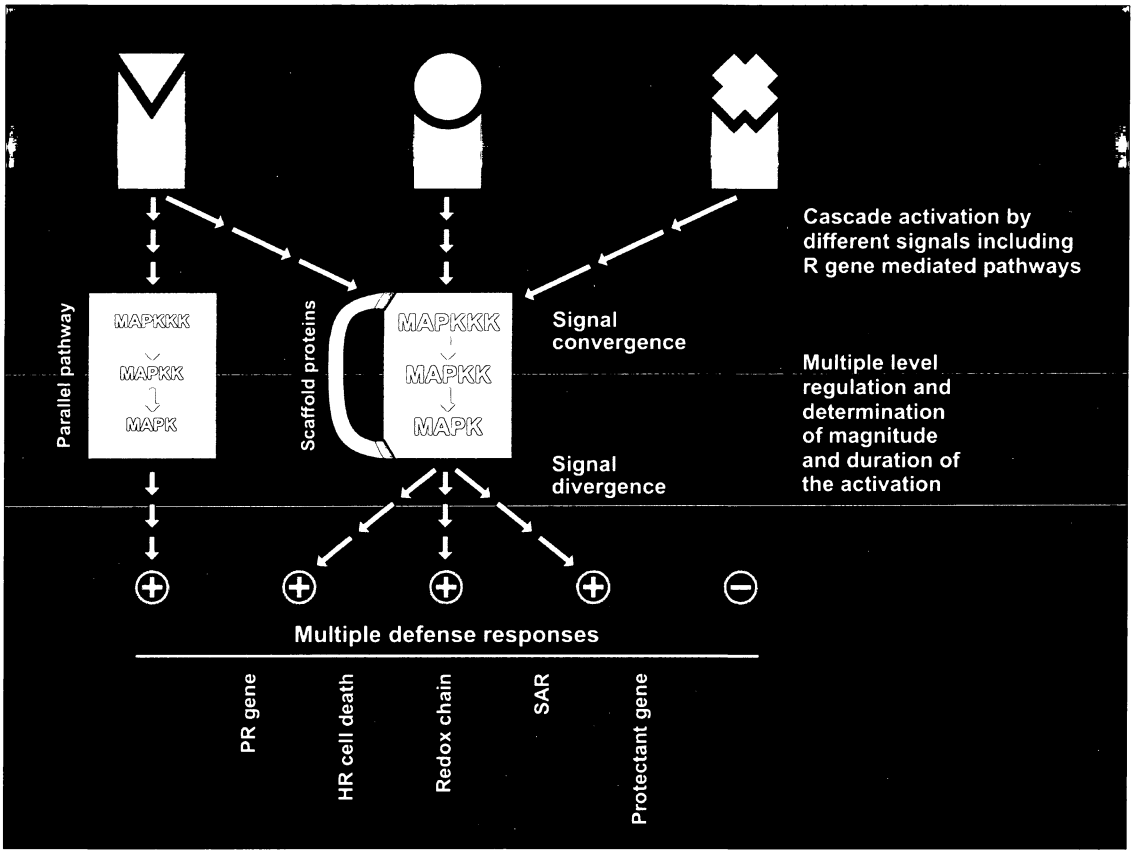
Protein kinases are able to amplify weak signals because one active kinase is able to phosphorylate hundreds of target proteins. Often specific protein kinases act in series, creating a protein kinase cascade. Protein kinases and kinase cascades have been implicated in a wide array of plant signal transduction pathways. The mitogen-activated protein kinase (MAPK) pathway is identified as one of the key phosphorylation pathways.

1.2 MAPK pathways in plants

MAPK cascade is a signaling system that plays essential roles in signal transduction processes involving cell proliferation, differentiation, death and stress responses in almost all eukaryotes. This cascade is formed by MAP kinase (MAPKs), MAPK kinase (MAPKKs) and MAPKK kinase (MAPKKKs). The MAPKKK-MAPKK-MAPK module is linked in various ways to upstream receptors and downstream targets. MAPKKK activation is mediated by receptor through physical interaction and/or phosphorylation by either receptor itself, intermediate bridging factors or interlinking MAPKKKs (Nakagami *et al.,* 2005). Studies have shown that both race- and non-race-specific pathogen-related elicitation activate MAPK cascades (Romeis *et al.,* 1999; Zhang and Klessig, 2000). MAPKKs are activated by MAPKKKs through phosphorylation on two serine/threonine residues in a conserved S/T-X_{3-5}-S/T motif. MAPKKs are dual-specificity kinases that phosphorylate MAPKs on threonine and tyrosine residues in the T-X-Y motif. MAPKs phosphorylate a variety of substrates including transcription factors, protein kinases and cytoskeleton-associated proteins (Nakagami *et al.,* 2005). In general, MAPK pathways bring together transduction pathways initiated by different types of signals and also deliver messages to diversified streams to evoke the transcription of different genes and to affect the effectiveness of downstream proteins (Romeis, 2001). The cascades components are bound together by scaffold proteins and they are regulated at multiple levels (i.e. transcriptional, translational and post-translational) (Xing *et al.*, 2002). The specificity of different MAPK cascades functioning within the same cell is generated through the presence of docking domains found in various components of MAPK modules and scaffold proteins. In addition, several MAPK pathways can be used in parallel by a single elicitation stimulus (Cardinale *et al.,* 2000; Kumar and Klessig, 2000). The downstream defense responses of a MAPK cascade might include the activation of pathogenesis-related (PR) genes (Hirt, 1997), hypersensitive response (HR)-like cell death (Yang *et al.,* 2001), redox chain (Grant *et al.,* 2000), systemic acquired resistance (SAR) and the activation of protective genes (Tena *et al.,* 2001). Figure 1-1 shows how MAPK cascades function and how they are regulated in plant defense (Xing *et al.*, 2002).

Increasing evidence has shown that MAPK cascade plays an important role in plant signal transduction related to biotic and abiotic stresses. It has been observed that MAPKs are activated in plants exposed to pathogens (Suzuki and Shinshi, 1995; Adam *et al.,* 1997; Ligterink *et al.,* 1997; Zhang and Klessig, 1997, 1998b; He *et al.,* 1999; Rudd *et al.*, 2008; Xing *et al.*, 2008), fungal elicitors (Suzuki and Shinshi, 1995), salicylic acid (Zhang and Klessig, 1997), jasmonic acid (Seo *et al.,* 1999), and abscisic acid (Knetsch *et al.,* 1996; Burnett *et al.,* 2000; Heimovaara-Dijkstra *et al.,* 2000). Many genes that are involved in MAPK cascades have been identified and studied. MEKK1 was one of the first MAPKKKs to be characterized in *Arabidopsis*; the gene is transcriptionally up-regulated in response to touch, cold, and salt stress (Mizoguchi *et al.,* 1996; Covic *et al.,* 1999). The most extensively characterized plant MAPKs are the tobacco salicylic-acid-induced protein kinase (SIPK; Zhang and Klessig, 1997) and the wound-induced protein kinase (WIPK; Seo *et al.*, 1995, 1999), and their orthologs in other plant species (Lee *et al.*, 2004; Pedley and Martin, 2005; Romeis, 2001; Zhang and Klessig, 2001). Studies suggest SIPK, WIPK, and NtMEK2 (*Nicotiana tabacum* MEK2, a tobacco MAPKK), an upstream kinase for both SIPK and WIPK, have a crucial function in induction of defense responses and hypersensitive cell death (Yang *et al.*, 2001; Zhang and Liu, 2001). The orthologs of these protein kinases in *Arabidopsis*, *Medicago sativa* and *Solanum tuberosum* have been suggested to have similar functions (Suzuki, 2002; Katou *et al.,* 2003). However, plants have more than 100 genes that encode MAPK-related proteins, yet relatively little is known about their function and contribution to different pathways (Takahashi *et al.*, 2007).

Figure 1-1: Dynamic regulatory mechanisms contribute to the final effectiveness of the mitogen-activated-protein kinase (MAPK) pathway in plant defense. (From Xing *et al.*, 2002).



1.3 Programmed cell death (PCD)

Programmed cell death (PCD) is a physiological cell death process involved in selective elimination of unwanted cells (Ellis *et al.*, 1991). It is recognized as a ubiquitous process of cellular suicide that lays dormant in all living cells from prokaryotes to eukaryotes (Beers and McDowell, 2001; Koonin and Aravind, 2002; Lewis, 2000; Madeo *et al.*, 2004; Watanabe and Lam, 2006). Terminal differentiation, senescence and disease resistance are the three main PCD research areas (Jones, 2001). PCD plays an important role in cell and tissue homeostasis and specialization, tissue sculpting, and disease resistance. One of the essential functions of cell death in plant development is that cells invaded by pathogens may be self eliminated as part of a hypersensitive response (HR) against the pathogen (Jones, 2001). PCD in plants as a response to pathogen attack is characterized by the generation of reactive oxygen species (ROS), activation of specific proteases, and fragmentation of DNA, eventually leading to HR (Lam *et al.,* 2001). This HR process is similar to apoptosis in animal cells (Lam *et al.,* 2001). Cell death signaling molecules such as ceramides can also regulate cell death in both animal and plant cells (Watanabe and Lam, 2006). These evidences argue for conserved cell death signaling mechanisms in eukaryotes, which provides plant researchers a way to study plant PCD by analogy to animal PCD molecules.

1.4 Genetic approaches

Several technologies have been successfully developed for the study of plant defense pathways. Microarray has been used to monitor expression patterns of 2375 selected genes in *Arabidopsis* after inoculation with fungal pathogen or treatment with the defense-related signaling molecules (Schenk *et al.*, 2000). Among nine MAPKs analyzed, eight were significantly induced by pathogen and four by salicylic acid, seven by ethylene and seven by MeJA (Schenk *et al.*, 2000; Xing *et al.*, 2002). The limitation of microarray and reverse transcription polymerase chain reaction (RT-PCR) is that they only detect transcriptional changes without the studies of translational regulation or post-translational modification (Xing *et al.*, 2002). Therefore, some approaches that can study

at protein level will be important, such as two-dimensional (2D) protein electrophoresis and western blotting. For example, Rudd *et al.* (2008) demonstrated that the wheat MAP kinase (TaMPK3) protein was post-translationally activated during the compatible interaction in parallel to PCD by performing western blotting.

Moreover, in order to identify and confirm *in vivo* target for specific kinases, some new technologies were adopted, including high-throughput yeast two-hybrid systems and TAP (tandem affinity purification)-tagging for protein-protein interaction study, fluorescence resonance energy transfer microscopy for microscopical detection on kinase-target interactions, and kinase chips for evaluating protein kinases on a large scale (Xing *et al.*, 2002).

By genetic approaches, MAPK genes with a role in disease resistance can be identified by the isolation and characterization of mutants. Many research groups also use the determination of differential gene expression to identify defense-related MAPK genes. Then, these identified genes can be studied by knock-out mutants and transgenic overexpression. In a previous work in our lab, constitutive activation of a tomato MAP kinase kinase gene, tMEK2, enhanced the expression of tomato PR and wound-related genes (Xing *et al.*, 2001). Transgenic tomato overexpressing the active form of tMEK2 became more resistant to virulant pathogen *Pseudomonas syringae* pv. *tomato* (Xing *et al.*, 2003), and transgenic wheat showed partial resistance to a virulant leaf rust pathogen (Fan *et al.*, 2009).

1.5 My project on wheat (*Triticum aestivum*)

Most characterized plant MAPK pathway components were isolated from dicot model species such *Arabidopsis,* tobacco, alfalfa and tomato, and our understanding of the role of MAPK cascades in stress response remains rather limited. Moreover, in economically important monocot species such as wheat, rice, maize or barley, there are very few MAPKs that have been identified and characterized (Yang and Xiong, 2008). As one of the most important food crops in the world, wheat is the most widely grown cereal crop

occupying 17% of all cultivated land worldwide and a staple food crop for 35% of the world's population providing 20% of calories consumed (Niu *et al.,* 2005). Wheat is considered as the backbone of Canadian agriculture. Disease is one of the major factors affecting wheat growth and development. A typical example is that fungal disease (such as leaf rust) causes serious wheat yield losses worldwide. Therefore, how to enhance the disease defense and how to increase the plant production are the essential challenges for researchers working on wheat.

Plant defense responses depend on recognition of invading pathogens, and this recognition may occur via gene-for-gene interactions in which a plant resistance (R) gene product detects the action of a cognate pathogen avirulence (Avr) factor (Nimchuk *et al.,* 2003). R-Avr interactions activate rapid immune responses at the infection site that are often mediated by salicylic acid (SA), and many virulent pathogens also activate basal resistance responses that involve SA (Broderson *et al.,* 2006). An immune state in systemic tissues termed systemic acquired resistance (SAR) is led by the activation of R proteins (Broderson *et al.,* 2006). The plant hormone salicylic acid, which is synthesized in a stress-dependent manner, is essential for many biotic stress responses (Koornneef and Pieterse, 2008). Early studies demonstrated the crucial role played by SA in mediating SAR by using transgenic tobacco expressing the bacterial *NahG* gene, which encodes an SA-degrading enzyme, since SA depletion impaired SAR induction (Gaffney *et al.,* 1993; Delaney *et al.,* 1994). Further studies supported these results by the analysis of the two SA-deficient *Arabidopsis* mutants *sid2* and *eds5*, which showed enhanced susceptibility to several bacterial pathogens (Rogers and Ausubel, 1997; Nawrath and Metraux, 1999). However, it has been suggested that some defense responses are SA-independent and under certain circumstances SA is not required. For instance, transgenic tobacco plants expressing a pokeweed antiviral protein exhibited resistance to a broad range of viruses in the absence of SA accumulation (Smirnov *et al.,* 1997). Therefore, it will be interesting to explore if SA can induce PCD in wheat and if MAPK components in wheat are involved in SA pathway.

Fumonisin B1 (FB1) is a toxin produced by the common corn fungus *Fusarium moniliforme*, which induces apoptosis-like PCD in both plants and animals (Asai *et al.*,

2000). This fungal toxin disrupts sphingolipid metabolism in eukaryotes by acting as a competitive inhibitor of ceramide synthase (Desai *et al.,* 2002), a key enzyme in sphingolipid biosynthesis (Abbas *et al.,* 1994; Yoo *et al.,* 1996; Stone *et al.,* 2000). Sphingolipids play diverse roles in many cellular processes, functioning both as anchors for membrane proteins (Futerman, 1995) and as secondary messengers regulating various cellular functions, including differentiation, growth and apoptosis (Spiegel and Merrill, 1996; Stone *et al.,* 2000). The sphingolipid ceramide, as a key component of the mammalian stress response pathway, activates several stress-activated protein kinases and phosphatases (Nickels and Broach, 1996; Zhang *et al.,* 1997; Stone *et al.,* 2000). Asai *et al.* (2000) have proposed a model for cell death signaling in *Arabidopsis* protoplasts induced by FB1.

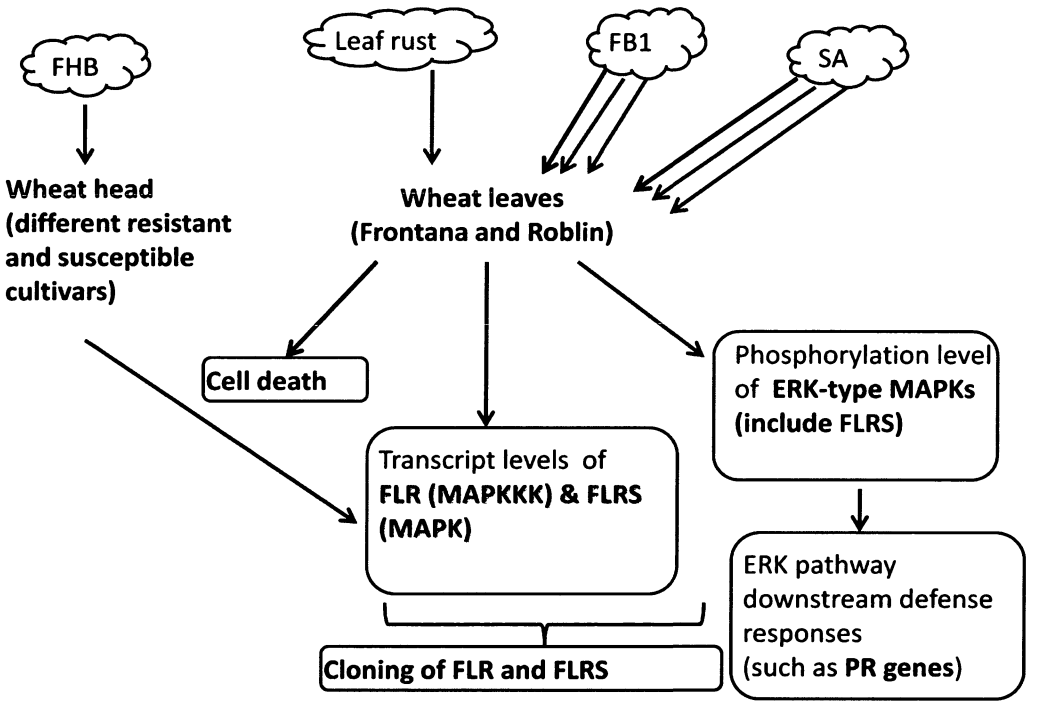
While studies using SA or purified fungal toxins provide us with information of focused defense response pathways, challenging host plants with pathogens verifies the involvement of the genes or proteins of interest in plant-pathogen interactions. Wheat leaf rust is caused by the fungus *Puccinia triticina* and is a common, worldwide problem resulting in wheat yield losses of up to 15% (Kolmer, 1996). The wheat-leaf rust interaction, which generally follows the gene-for-gene model where a pathogen-encoded avirulence (Avr) gene overcomes a host-encoded resistance (R) gene (Flor, 1971), remains an excellent system to study fungal diseases of plants, and many of the *Lr* genes are studied for this reason. Genetic resistance is the preferred method to reduce losses from leaf rust, and 60 leaf rust resistance (Lr) genes have been designated in wheat (McIntosh *et al.,* 2007). The presence of single leaf rust resistance genes in near-isogenic lines confers resistance to avirulent races resulting in a hypersensitive response at the site of infection with localized cell death, and significant amelioration of disease symptoms (Kolmer, 1996). This plant-pathogen interaction has been long considered a model system in the analysis of wheat defense pathways (Jordan *et al.*, 2006).

Fusarium head blight (FHB) caused by *Fusarium graminearum* Schwabe [teleomorph *Gibberella zeae* (Schwein.) Petch] is a devastating disease of wheat (*Triticum aestivum* L.) and other small grains (Gilbert and Tekauz, 2000). Wheat infection by FHB results in significant yield losses and severe grade penalties (Gilbert and Tekauz, 2000).

Furthermore, the most serious threat of FHB is the problem of grain contamination caused by associated mycotoxins such as deoxynivalenol (DON), zearalenone and moniliformin (Yang *et al.,* 2006). FHB resistance in wheat is inherited quantitatively and there are multiple genes acting in combination to provide the maximum level of resistance. Complicating this pathosystem further is the fact that FHB resistance is characterized by several mechanisms, including reduced initial infection, reduced spread of the disease, and reduced levels of mycotoxins, all of which may be controlled by separate gene loci (Gilbert *et al.,* 2006).

For my Master thesis, I am working on two wheat MAPK pathway genes. FLR (GenBank accession number: AY173961) is predicated as a MAPKKK, and FLRS (GenBank accession number: AY173962) is predicated as a MAPK. In the thesis, the following work will be presented: (1) Bioinformatics analysis of these two genes and the cloning work of FLRS; (2) The analysis of gene expression level of FLR and FLRS under biotic stresses by RT-PCR and Northern blotting; (3) The study on post-translational regulation of ERK-type MAPKs under biotic stresses by western blotting; (4) Confirmation on if ERK-type MAPKs are involved in SA pathway by using an ERK specific inhibitor. (5) Microscopic study of PCD on both FHB susceptible and resistant wheat cultivars under biotic stresses. Figure 1-2 describes my project overview.

Figure 1-2: Overview of my project on wheat MAPK pathway genes. Blue arrows indicate that wheat will be treated with FHB, leaf rust, FB1 and SA, and FLR and FLRS transcript levels will be examined. Green arrows present that phosphorylation level of ERK-type MAPKs (include FLRS) will be studied when wheat leaves are treated with FB1 and SA, and ERK pathway downstream defense responses (such as PR genes) will be also studied. Black arrows indicate that cell death on wheat leaves under FB1 and SA treatment will be detected. The red arrow shows that FLR and FLRS will be tried to be cloned.



Chapter II

Transcriptional Changes of Wheat FLR and FLRS Genes during Biotic Stresses

2.1 Introduction

Plants encounter a vast array of pathogenic micro-organisms in their natural environment, and these diverse pathogens deliver virulence factors into the plant cell to promote virulence and cause disease (Jones, 2009). Plants protect themselves against microbes and disease through defense mechanisms, which are usually complex and composed of multiple layers of defense (Jones, 2009). Plants have evolved a wide variety of inducible defense mechanisms that are triggered upon pathogen recognition. Expression of defense-related genes is one of these inducible defenses.

Mitogen-activated protein kinases (MAPKs) are components of signaling pathways that transduce diverse extracellular stimuli to multiple intracellular responses (Seo *et al.,* 2007). Studies have shown that MAPK cascades play important roles in plant defense to multiple stresses (Zhang and Klessig, 2001). Some of the early studies demonstrated that the involvement of plant MAPKs in stress signaling was the transcriptional activation of wound-induced protein kinase (WIPK) by wounding (Seo *et al.,* 1995). Later evidence indicated that the WIPK transcript was induced by various elicitors and pathogens (Zhang and Klessig, 1998; Zhang *et al.,* 2000; Romeis *et al.*, 1999). In wheat, MAPK TaMPK3 which is a WIPK-like kinase, was strongly transcriptionally up-regulated during the immediate presymptomatic phase of the compatible interaction (Rudd *et al.,* 2008). Also, MAP kinase kinase 4 (MKK4) from alfalfa (*Medicago sativa*), MAP kinase 3 (MPK3) from *Arabidopsis* and elicitor responsive MAP kinase (ERMK) from parsley are induced likewise at the mRNA level (Jonak *et al.,* 1996; Bogre *et al.,* 1997; Mizoguchi *et al.,* 1996; Ligterink *et al.,* 1997). A gene encoding a MAP kinase in wheat, TaWCK1 is activated transcriptionally by a fungal elicitor (Takezawa, 1999). The transcripts of the gene encoding *Medicago sativa* MAP kinase homolog named MsTDY1 and *Oryza sativa*

blast- and wound-induced MAP kinase (OsBWMK1) are induced by wounding and pathogen infection as well (Schoenbeck *et al.,* 1999; He *et al.,* 1999). Currently, Northern blotting analysis revealed that the transcripts of GhMAPK (a novel MAP kinase gene in cotton) accumulated markedly when the cotton seedlings were subjected to various abiotic stimuli, and GhMAPK was upregulated by the exogenous signaling molecules, such as salicylic acid and hydrogen peroxide, as well as pathogen attacks (Wang *et al.,* 2007). Moreover, Wang *et al.* (2010) revealed that the transcripts level of *ZmMPK3* (a novel mitogen-activated protein kinase gene in maize) accumulated markedly and rapidly when maize seedlings were subjected to exogenous signaling molecules and various abiotic stimuli. These results suggest that stress-induced transcription of genes for MAPKs play an important role in plant defense responses.

In this project, we have studied two wheat genes, FLR and FLRS. FLR is identified as a MAPKKK, and FLRS is identified as a MAPK by bioinformatics analysis. Therefore, FLR and FLRS are hypothesized as plant defense-related genes in this project. The other reason for our hypothesis is that these two genes were discovered when we found they responded to wheat leaf rust challenge in preliminary wheat expressed sequence tag (EST) array analysis (He and Xing. unpublished). Wheat (*Triticum aestivum*) EST database, which was established in a genome sequencing project in Genomics Initiative of Agriculture and Agri-Food Canada (AAFC) (First and second competition), was used for sequence assembly in 2002 (Travis Banks, AAFC). During EST array analysis, only singleton database was used to do the sequence assembly (UNIGENE set was not available at the time). Both sequences were deposited to GenBank in 2002.

SA is known to play a major role in plant defense and is generally involved in the activation of defense responses (Grant and Lamb, 2006). Alteration of SA level in plant results in the change in the expression of defense related genes and activation of defense response (Jones, 2009). Also, it has been suggested that FB1 induces MAPK activity (Pinelli *et al.,* 1999), and the activation of MAPK induced some effects, including transcription, modulation and activation of enzymes involved in cell signal transduction. Therefore, studies on FLR and FLRS using SA and FB1 may provide us with more information of defense response pathways in wheat.

The wheat-leaf rust interaction generally follows the Avr gene-for-R gene model, so this is an excellent model to study if FLR and FLRS are involved in the resistance to fungal pathogens. Marcel *et al.* (2007) showed that a MAPKKK in barley contributes to partial resistance to barley leaf rust. Furthermore, fusarium head blight (FHB), which is a serious disease of wheat worldwide that may cause substantial yield and quality losses (Yang *et al.,* 2006), is also used as a pathogen challenge on wheat in this project. The development of genetically FHB-resistant cultivars is the most cost-effective method to control the disease (Yang *et al.,* 2006).

In this chapter, we have identified that the two wheat genes, FLR and FLRS, are MAPKKK and MAPK respectively by informatics analysis. Then we studied the possible involvement of FLR and FLRS in defense response pathways by treating wheat with SA and purified fungal toxin FB1. Also, the host plants are challenged by leaf rust and FHB in order to verify the involvement of these two genes in plant-pathogen interactions. Both of FLR and FLRS transcript levels are not changed in SA and FB1 treatments, suggesting that these two genes may not play a role in the SA-mediated pathway and in response to FB1 in transcriptional level. On the other hand, FLR and FLRS expressions are significantly up-regulated during leaf rust and FHB challenges, verifying that they are involved in plant-pathogen interactions. Therefore, we try to clone FLR and FLRS, and overexpress them in wheat.

2.2 Results

2.2.1 Identification of FLR and FLRS

Full-length cDNA sequence and the deduced amino acid sequence of FLR are showed in Figure 2-1. FLR has the signature motif 'GTPEFMAPE' (shaded in yellow), which is for one of the MAPKKK classes (ZIK1 class) (Cvetkovska *et al.*, 2005) and amino acid residues 30 through to 301 are identified as catalytic domains of protein kinases. This sequence is also identified to be serine/threonine protein kinase-related by using InterProScan Sequence Search tool (Figure 2-1). Analysis with ExPASY SIM-Alignment tool has indicated that 47.6% of amino acid residues in this catalytic domain are identical

in *Triticum aestivum* FLR (AY173961) and *Homo sapiens* putative mitogen-activated protein kinase kinase kinase (PRKWNK2 gene) (AJ242724) (Figure 2-2).

Nucleotide sequence and the deduced amino acid sequence of FLRS are presented in Figure 2-3. FLRS has one T-X-Y motif shaded in yellow (Figure 2-3), and MAPKKs phosphorylate MAPKs on threonine and tyrosine residues in the T-X-Y motif. Amino acids 62 to 357 are identified as the protein kinase domain using InterProScan Sequence Search tool (Figure 2-3). Amino acids sequence alignment has been done between *Triticum aestivum* mitogen-activated protein kinase (FLRS) (AY173962) and MAP kinase 6 from *Oryza sativa* (japonica cultivar-group) Os06g0154500 (NM_001063381) (Figure 2-4). Alignment result in Figure 2-4 has indicated that FLRS and *Oryza sativa* MAPK have 91.4% identity in 405 residues overlap.

Figure 2-1: Nucleotide sequence and the deduced amino acid sequence of wheat FLR gene. The signature motif for one of MAPKKK classes (ZIK1 class) is shaded in yellow. The stop codon is indicated by asterisk. Highly conserved catalytic domain of protein kinases is underlined.

```
   1 atgtcgagcccgcggcggccggagatgtcaccggcggcgaggaacagcgacaatggatac  60
     M  S  S  P  R  R  P  E  M  S  P  A  A  R  N  S  D  N  G  Y
  61 gtcgagaccgaccccactggtcgctacggccggttcgacgagctcctgggcaaggggcg  120
     V  E  T  D  P  T  G  R  Y  G  R  F  D  E  L  L  G  K  G  A
 121 atgaagtcggtgtacaggggttcgacgaggtgcgcggcgtggaggtggcgtggaaccag  180
     M  K  S  V  Y  R  G  F  D  E  V  R  G  V  E  V  A  W  N  Q
 181 gccaacctcgccgacgtcctccgcacccccgacgccctgcagcgcatgtactccgaggtc  240
     A  N  L  A  D  V  L  R  T  P  D  A  L  Q  R  M  Y  S  E  V
 241 cacctcctcagcacgctccgccacgacgccatcatcgccttccacgcctcatgggtctcc  300
     H  L  L  S  T  L  R  H  D  A  I  I  A  F  H  A  S  W  V  S
 301 gtctcctccccctccccgcgcggcggctgcaccggcggcaccccacgccgcaccttcaac  360
     V  S  S  P  S  P  R  G  G  C  T  G  G  T  P  R  R  T  F  N
 361 ttcatcaccgagctcttctcctccggcaccctccgcgcgtaccgcctccggtacccgcgc  420
     F  I  T  E  L  F  S  S  G  T  L  R  A  Y  R  L  R  Y  P  R
 421 gtgagcctccgcgccgtccgcggctgggcgcgccagatcctccggggcctcgcctacctc  480
     V  S  L  R  A  V  R  G  W  A  R  Q  I  L  R  G  L  A  Y  L
 481 cacgcccacgacccccccgtcatccaccgcgacctcaagtgcgacaacgtgttcgtcaac  540
     H  A  H  D  P  P  V  I  H  R  D  L  K  C  D  N  V  F  V  N
 541 ggccaccagggcaccgtcaagatcggcgacctcggcctcgccgccgtgctccgcggcgcc  600
     G  H  Q  G  T  V  K  I  G  D  L  G  L  A  A  V  L  R  G  A
 601 caggccgcgcacagcgtcatcggcacgcccgagttcatggcgcccgagatgtacgacgag  660
     Q  A  A  H  S  V  I  G  T  P  E  F  M  A  P  E  M  Y  D  E
 661 gactacgacgagctcgtcgacgtctactccttcggcatgtgcatgctcgagatgctcacc  720
     D  Y  D  E  L  V  D  V  Y  S  F  G  M  C  M  L  E  M  L  T
```

721 gtcgagtacccctacgccgagtgctccaaccccgcgcagatctacaagaaggtcacctcc 780

V E Y P Y A E C S N P A Q I Y K K V T S

781 ggcaagctgcctgacgccttctaccgggtcgatgacgccgacgcgcgcaggttcatcggc 840

G K L P D A F Y R V D D A D A R R F I G

841 aggtgccttgtccccgcctcccatcgcccctccgcccaggagctcctgctcgaccgtttc 900

R C L V P A S H R P S A Q E L L L D R F

901 ctctccacgcaggacaccaccatgacactgtcacctccgccattgctgcctgccttgccc 960

L S T Q D T T M T L S P P P L L P A L P

961 acctccggtgaccggaaggataacccggaggaggctgagccggtggcagcaaggactgac 1020

T S G D R K D N P E E A E P V A A R T D

1021 atgaccatcaccggcaagctcaacaccgacgacgacaccatcttcctcaaagtgcagatc 1080

M T I T G K L N T D D D T I F L K V Q I

1081 gtcgacgaagcaggccattcgaggaacatctacttcccgttcgacatcgccggcgacaca 1140

V D E A G H S R N I Y F P F D I A G D T

1141 gcgacggaggtggcgagggagatggtgaaggagctggacatcacggaccgggacccctca 1200

A T E V A R E M V K E L D I T D R D P S

1201 gagatcgccgccatgatcgagcaggagatcacgaggctggtaccggactgggtcggcggt 1260

E I A A M I E Q E I T R L V P D W V G G

1261 ggctgtgatgatcagcaggagtactatacgtacgccgataatgacgacaatgaggagcag 1320

G C D D Q Q E Y Y T Y A D N D D N E E Q

1321 cctcctttctactacctctcctcttccccgacctcttcaaacgggtctcattgtggcaca 1380

P P F Y Y L S S S P T S S N G S H C G T

1381 gggccgacaacatcaggtggcggctacgctggctggttccaagattacgctgtgagcagc 1440

G P T T S G G G Y A G W F Q D Y A V S S

1441 gacgacgacgagacgagctccacgcgctccgcgctgcactactcctccgaagaggcccag 1500

D D D E T S S T R S A L H Y S S E E A Q

1501 cccgaagagaagcctggcgtctccaagacgggccaggtgaaggccaccagattcggccca 1560

P E E K P G V S K T G Q V K A T R F G P

```
1561 ggagacagcggcacagcaggacacgacgtgtcgtcgtcgcgcgccggccggccgcggcac 1620
     G  D  S  G  T  A  G  H  D  V  S  S  S  R  A  G  R  P  R  H
1621 caccgcgggagccccgacgccggcggcgacgaggggcgcccgcggaggcagcagggccgc 1680
     H  R  G  S  P  D  A  G  G  D  E  G  R  P  R  R  Q  Q  G  R
1681 atgacgaggaaccggtcgatggtggacgtgcggagccagctgctgcaccggacgctggtg 1740
     M  T  R  N  R  S  M  V  D  V  R  S  Q  L  L  H  R  T  L  V
1741 gaggagctcaacaagcgcatgttcttcaacaccgtcggcgcggtcgagaacatcggcttc 1800
     E  E  L  N  K  R  M  F  F  N  T  V  G  A  V  E  N  I  G  F
1801 cgccgcatccccggctacggcggcgggccctcgtcctcgtcggcgacggtgacgtcctcg 1860
     R  R  I  P  G  Y  G  G  G  P  S  S  S  S  A  T  V  T  S  S
1861 cgcgggggcgaccagcgcggccggagaagtggcaaggacaagcaccagttcttcatgttc 1920
     R  G  G  D  Q  R  G  R  R  S  G  K  D  K  H  Q  F  F  M  F
1921 tga
     *
```

Figure 2-2: Alignment of wheat FLR (AY173961) and *Homo sapiens* mitogen-activated protein kinase kinase kinase (PRKWNK2 gene) (AJ242724) amino acid sequences using ExPASy SIM-Alignment tool. Identities = 47.6% identity in 311 residues overlap.

```
AY173961,     25 VETDPTGRYGRFDELLGKGAMETKSVYRGFDEVRGVEVAWNQANLADVLRTPDALQRMET
AJ242724,    192 VATSLDGRFLKFDIELGRGSFKT--VYKGLDTETWVEVAW--CELQDRKLTKLERQRFKE
                 * *   **   **  ** *   *  ** * *    *****    * *   *    **

AY173961,     85 YSEVHLLSTLRHDAIIAFHASWVSVSSPSPRGGCTGGTPRRTFNFITELF--SSGTLRAY
AJ242724,    248 EAEMETLKGLQHPNIVRFYDFWES----SAKG-------KRCIVLVTELMETTSGTLKTY
                   *   *  * *  *  *   * *    *  *        *     ***    ****  *

AY173961,    143 --RLRYPRVSLRAVRGWARQILRGLAYLHAHDPPVIHRDLKCDNVFVNGHQGTVKIGDLG
AJ242724,    297 LKRFKVMETKPKVLRSWCRQILKGLLFLHTRTPPIIHRDLKCDNIFITGPTGSVKIGDLG
                   *           * * **** **  **   ** ********* *  *  * *******

AY173961,    201 LAAVLRGAQAAHSVIGTPEFMETAPEMETYDEDYDELVDVYSFGMETCMETLEMETLTVE
AJ242724,    357 LAT-LKRASFAKSVIGTPEFMETAPEMETYEEHYDESVDVYAFGMETCMETLEMETATSE
                 **  *  *  * ****************** * *** **** ************** * *

AY173961,    261 YPYAECSNPAQIYKKVTSGKLPDAFYRVDDADARRFIGRCLVPASHRPSAQELLLDRFLS
AJ242724,    416 YPYSECQNAAQIYRKVTCGIKPASFEKVHDPEIKEIIGECICKNKEERYEIKDLLSHAFF
                 *** ** * **** *** *  *  *  * *      ** *             **

AY173961,    321 TQDTTMETTLS
AJ242724,    476 AEDTGVRVELA
                   **     *
```

Figure 2-3. Nucleotide sequence and the deduced amino acid sequence of FLRS. T-X-Y motif is shaded in yellow. The stop codon is indicated with asterisk. Highly conserved catalytic domain of protein kinases is underlined.

```
  1 atggacgccggcggggcgcagccgccggactcggagatggcggaggccggtgcgggcgct 60
    M  D  A  G  G  A  Q  P  P  D  S  E  M  A  E  A  G  A  G  A
 61 gcggcggcggcggggacggcgccgggcggcgcgatggataacatccaggccacgctcacc 120
    A  A  A  A  G  T  A  P  G  G  A  M  D  N  I  Q  A  T  L  T
121 cacggcgggaggttcatccagtacaacatcttcggcaacgtcttcgaggtcaccgccaag 180
    H  G  G  R  F  I  Q  Y  N  I  F  G  N  V  F  E  V  T  A  K
181 tacaagccccccatcctccccatcggcaagggcgcctacggcatcgtctgctccgcgctc 240
     Y  K  P  P  I  L  P  I  G  K  G  A  Y  G  I  V  C  S  A  L
241 aactccgagacgggggagcaggtggccatcaagaagatcgccaacgccttcgacaacaag 300
    N  S  E  T  G  E  Q  V  A  I  K  K  I  A  N  A  F  D  N  K
301 atcgacgccaagcgcacgctgcgggagatcaagctgctccgccacatggaccacgagaat 360
    I  D  A  K  R  T  L  R  E  I  K  L  L  R  H  M  D  H  E  N
361 attgttgcaataagggatattatacctcctgcacaaaggactgcattcaatgatgtctat 420
    I  V  A  I  R  D  I  I  P  P  A  Q  R  T  A  F  N  D  V  Y
421 attgcatatgaattgatggacaccgatctgcatcaaattattcgctcaaatcaagcttta 480
    I  A  Y  E  L  M  D  T  D  L  H  Q  I  I  R  S  N  Q  A  L
481 tcggaggagcactgccagtatttcctttatcagatccttcgtggcttgaagtacatacat 540
    S  E  E  H  C  Q  Y  F  L  Y  Q  I  L  R  G  L  K  Y  I  H
541 tcagcaaatgttctccaccgagacttgaagcctagcaatcttcttttgaatgcaaactgt 600
    S  A  N  V  L  H  R  D  L  K  P  S  N  L  L  L  N  A  N  C
601 gacctaaaaatttgcgattttgggcttgctcgtaccacctcagaaactgattttatgacc 660
    D  L  K  I  C  D  F  G  L  A  R  T  T  S  E  T  D  F  M  T
661 gagtatgttgtgacaagatggtacagggcaccagagcttttgttgaactcctccgaatac 720
    E  Y  V  V  T  R  W  Y  R  A  P  E  L  L  L  N  S  S  E  Y
```

721 actgcagcaattgatgtgtggtctgtgggctgtatatttatggaactgatggaccggaaa 780

T A A I D V W S V G C I F M E L M D R K

781 ccgttgtttccaggaagagaccatgtccatcagctacgtctactaatggaggttttccct 840

P L F P G R D H V H Q L R L L M E V F P

841 tgtggtatttcctcgttgcagctcattggaacaccaaatgaggccgatttggattttgta 900

C G I S S L Q L I G T P N E A D L D F V

901 aatgaaaatgcaagaagatatatccgccaacttccccgtcatgcaaggcaatcattatct 960

N E N A R R Y I R Q L P R H A R Q S L S

961 gagaagtttccacatgttcacccttcagcaattgacttggttgaaaagatgctgactttc 1020

E K F P H V H P S A I D L V E K M L T F

1021 gatcctagacagagaataacagttgaaggcgcacttgcgcatccttacctggcatcgctg 1080

D P R Q R I T V E G A L A H P Y L A S L

1081 catgatataagtgatgagccagtctgcacgatgcccttcagctttgacttcgagcagcat 1140

H D I S D E P V C T M P F S F D F E Q H

1141 gcgttgtcggaagaacaaatgaaggatctaatccaccaggagggcatcacgttcaaccct 1200

A L S E E Q M K D L I H Q E G I T F N P

1201 gattaccagtag

D Y Q *

Figure 2-4. Amino acid sequence alignment of wheat FLRS (AY173962) and MAP kinase 6 from *Oryza sativa* (japonica cultivar-group) Os06g0154500 (NM_001063381). 91.4% identity in 405 residues overlap.

```
AY173962,      5 GAQPPDSEMAEAGAG-----AAAAAGTAPG-GAMDNIQATLTHGGRFIQYNIFGNVFEVT
NM_0010633     4 GAQPPDTEMAEAGGGQQPPAAAAAAGAGAGAGMMENIQATLSHGGRFIQYNIFGNVFEVT
                 ****** ****** *     ******   * * * ****** ******************

AY173962,     59 AKYKPPILPIGKGAYGIVCSALNSETGEQVAIKKIANAFDNKIDAKRTLREIKLLRHMDH
NM_0010633    64 AKYKPPILPIGKGAYGIVCSALNSETGEQVAIKKIANAFDNKIDAKRTLREIKLLRHMDH
                 ************************************************************

AY173962,    119 ENIVAIRDIIPPAQRTAFNDVYIAYELMDTDLHQIIRSNQALSEEHCQYFLYQILRGLKY
NM_0010633   124 ENIVAIRDIIPPPQRNSFNDVYIAYELMDTDLHQIIRSNQALSEEHCQYFLYQILRGLKY
                 ************ **  ******************************************

AY173962,    179 IHSANVLHRDLKPSNLLLNANCDLKICDFGLARTTSETDFMTEYVVTRWYRAPELLLNSS
NM_0010633   184 IHSANVLHRDLKPSNLLLNANCDLKICDFGLARTTSETDFMTEYVVTRWYRAPELLLNSS
                 ************************************************************

AY173962,    239 EYTAAIDVWSVGCIFMELMDRKPLFPGRDHVHQLRLLMEVFPCGISSLQLIGTPNEADLD
NM_0010633   244 EYTAAIDVWSVGCIFMELMDRKPLFPGRDHVHQLRLLME----------LIGTPNEADLD
                 ************************************          ***********

AY173962,    299 FVNENARRYIRQLPRHARQSLSEKFPHVHPSAIDLVEKMLTFDPRQRITVEGALAHPYLA
NM_0010633   294 FVNENARRYIRQLPRHARQSFPEKFPHVHPLAIDLVEKMLTFDPRQRITVEGALAHPYLA
                 ******************  ******** ****************************

AY173962,    359 SLHDISDEPVCTMPFSFDFEQHALSEEQMKDLIHQEGITFNPDYQ
NM_0010633   354 SLHDISDEPVCSSPFSFDFEQHALSEEQMKDLIYQEGLAFNPDYQ
                 ***********  ******************* ***  ******
```

2.2.2 No change of FLR and FLRS transcript levels with SA or FB1 treatment

MAPK cascades activated by several R gene-mediated pathways and pathogen-derived elicitors play an essential role in plant defense against pathogen attacks (Zhang and Klessig, 2001), suggesting FLR and FLRS, which have been identified as components of MAPK cascades might be involved in plant responses to stresses or hormones. It has been known that SA is a crucial signal molecule leading to systemic acquired resistance (SAR) and PR gene induction (Delaney *et al.,* 1994; Gaffney *et al.,* 1993), and also plays a role in regulation of gene expression in the course of defense response (Morris *et al.,* 2000). FB1 is naturally produced by *Fusarium spp*, including *F. moniliforme* that may play a role in virulence (Asai *et al.,* 2000; Dutton, 1996; Gilchrist, 1997; Jardine and Leslie, 1999), and elicits an apoptotic form of PCD in both plants and animal tissue culture cells (Tolleson *et al.,* 1996; Wang *et al.,* 1996a, 1996b; Yoo *et al.,* 1996; Gilchrist, 1997; Stone *et al.,* 2000). Therefore, we use FB1 as a biotic stress in this project. To determine whether FLR and FRLS are associated with SA-dependent pathway, and if these two genes play a role in response to biotic stress FB1, we carried out RT-PCR to monitor the transcriptional changes of FLR and FLRS genes upon SA and FB1 treatments. As MAPK cascades mediate plant innate immune responses (Asai *et al.*, 2001), it is expected the signalling events in leaves may also represent those in wheat head during FHB attack. Therefore, leaf tissues were used in this study.

RT-PCR analysis has revealed that there are no changes of FLR and FLRS transcript levels when wheat plants (Frontana and Roblin) were treated with SA and FB1, since the band intensity ratios of FLR and FLRS to wheat actin gene are all around 1 (Table 2-1). Wheat actin is the housekeeping gene that does not change during stresses (Mackintosh *et al.,* 2007).

Table 2-1: The band intensity ratios of FLR and FLRS to actin in Frontana and Roblin cultivars with SA and FB1 treatments (from RT-PCR analysis). This table presents one group of data, and the 2nd repeat experiment gave the similar result.

Wheat cultivars	Treatment	Incubation time (hour)	Band intensity ratio, FLR/Actin	Band intensity ratio, FLRS/Actin
Frontana	SA	0	1.00	1.00
	SA	24	0.90	0.95
	SA	48	1.01	0.97
	SA	72	0.89	0.98
	FB1	0	1.00	1.00
	FB1	36	1.06	1.05
	FB1	72	0.95	1.02
Roblin	SA	0	1.00	1.00
	SA	24	0.97	0.97
	SA	48	0.96	1.01
	SA	72	1.04	1.00
	FB1	0	1.00	1.00
	FB1	36	1.05	1.08
	FB1	72	0.94	0.97

2.2.3 Up-regulation of FLR and FLRS transcripts by leaf rust challenge

Wheat near isogenic line Thatcher Lr16 was inoculated with the leaf rust pathogen, race BBB or race TJB. The activation of plant defense responses is initiated upon plant-pathogen recognition, mediated either by a gene-for-gene interaction between a plant resistance (R) gene and pathogen avirulence (Avr) gene, or by the binding of a non race-specific elicitor to the receptor (Baker *et al.,* 1997; Dangl and Jones, 2001; Hammond-Kosack and Jones, 1996). Race BBB, an incompatible leaf rust challenge, has the Avr gene to react with the product of Lr16 R gene. Race TJB, a compatible leaf rust challenge, doesn't have Avr gene that can be recognized by Lr16, so there is no gene-for-gene interaction.

In order to verify if FLR and FLRS are involved in pathogen resistance, Northern blotting was performed to detect the transcript levels of these two genes upon leaf rust challenge. Northern blot results have shown that both FLR and FLRS are transcriptionally up-regulated upon Lr16-BBB incompatible interaction by comparing with Lr16 control and Lr16-TJB compatible interaction at 1.5 hours (Figure 2-5 and Figure 2-6). At 4 hours, there are no changes on FLR and FLRS transcriptional level comparing to control (Figure 2-5 and Figure 2-6). 25S rRNA exhibited most stable expression in plants grown under various environmental conditions (Jain *et al.,* 2006), so 25S rRNA is chosen as the control gene for equal RNA loading.

Figure 2-5. Transcriptional up-regulation of FLR gene in wheat upon leaf rust challenge (detected by Northern blotting). Wheat near isogenic line Thatcher Lr16 was inoculated with the leaf rust pathogen, race BBB or race TJB. 25S rRNA is the control gene for equal RNA loading. The experiments were repeated three times with similar results.

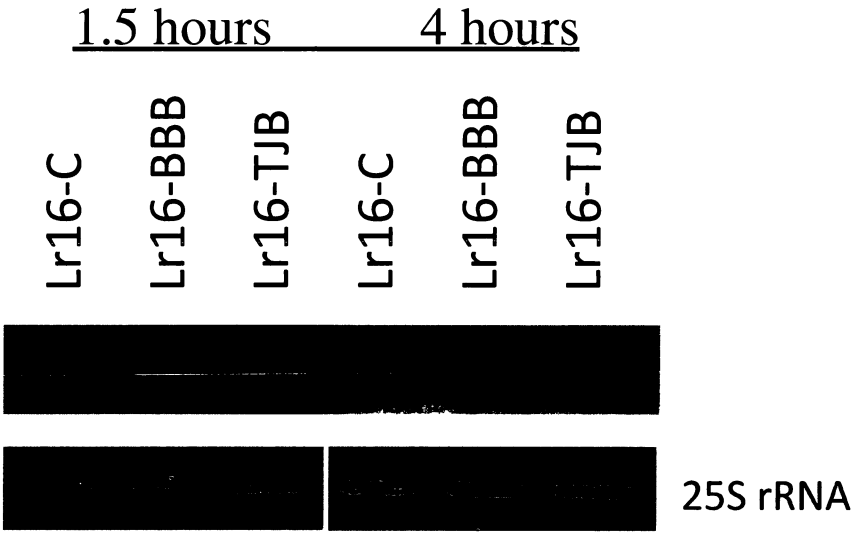
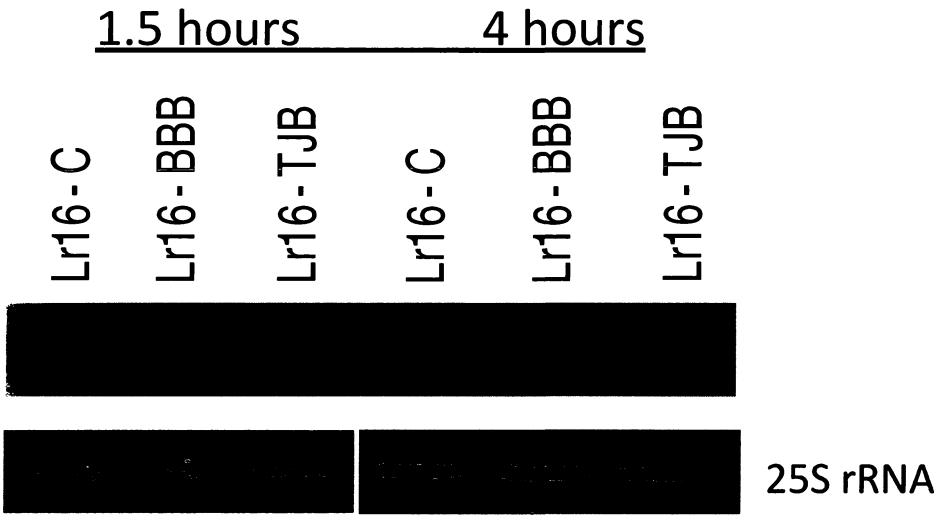


Figure 2-6. Transcriptional up-regulation of FLRS gene in wheat upon leaf rust challenge (detected by Northern blotting). Wheat near isogenic line Thatcher Lr16 was inoculated with the leaf rust pathogen, race BBB or race TJB. 25S rRNA is the control gene for equal RNA loading. The experiments were repeated three times with similar results



2.2.4 Up-regulation of FLR and FLRS transcripts by *Fusarium graminearum* challenge

Susceptible and resistant wheat cultivars were challenged by *Fusarium graminearum* the causal agent of fusarium head blight. The transcriptional levels of FLR and FLRS were detected by Northern blot. Upon fusarium head blight, FLR gene is activated in Frontana (a resistant cultivar) but not in Roblin (a susceptible cultivar). However, the background level of FLR in Roblin was high (Figure 2-7).

In order to find out the correlation between the mRNA level of FLR and resistance/susceptibility, Northern blotting was performed to detect background transcriptional levels of FLR gene in different resistant and susceptible cultivars (Figure 2-8). The result has suggested that there is no clear correlation between FLR transcript level and resistance or susceptibility to FHB (Figure 2-8).

The transcript level of FLRS gene in wheat head was very low and infection with FHB did not activate this kinase gene promptly until 24 hours after the challenge (Figure 2-9). At 6 hours, FLRS transcript level was not changed in either Roblin or Frontana, and the black dots are background. At 24 hours, FLRS was up-regulated in Frontana in response to FHB (red arrow).

Figure 2-7: Activation of wheat FLR by fusarium head blight challenge (analyzed by Northern blotting). 25S rRNA is the control gene for equal RNA loading. The experiments were repeated three times with similar results.

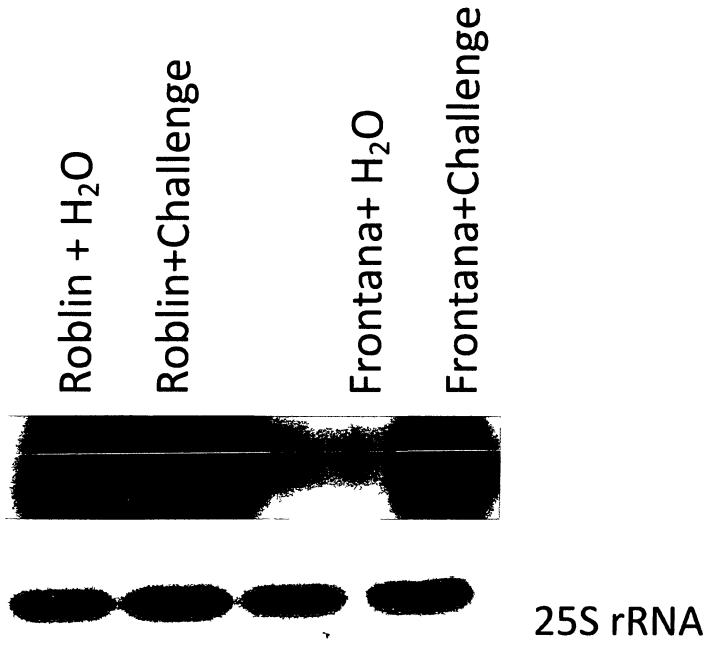


Figure 2-8: Background transcriptional levels of FLR gene in different resistant and susceptible cultivars (analyzed by Northern blotting). The experiments were repeated twice with similar results.

R- Resistant cultivar; S- Susceptible cultivar.

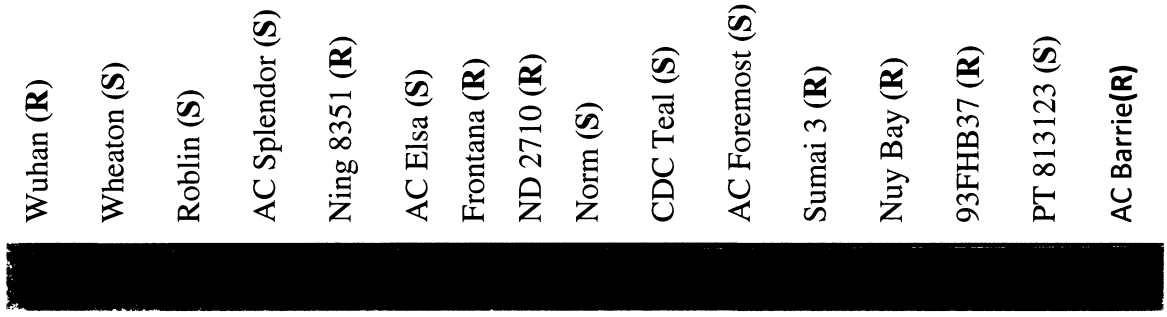
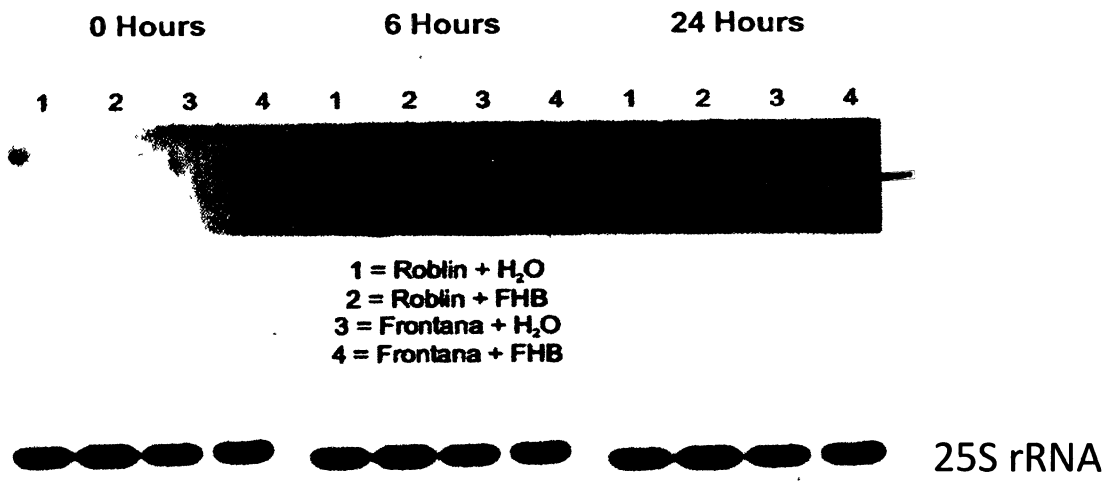


Figure 2-9: Transcriptional changes of FLRS gene in response to fusarium head blight challenge (detected by Northern blotting). 25S rRNA is the control gene for no change of RNA loading. Activation of FLRS gene at 24 hours is indicated by red arrow. The experiments were repeated three times with similar results.



2.2.5 Cloning of FLR

As shown in section 2.2.3 and 2.2.4, FLR is activated by leaf rust and FHB challenges, so it would be interesting to see if transgenic plants overexpressing FLR have different disease resistant properties compared to wild type wheat plants.

The first step of cloning is to amplify FLR by PCR. The FLR_F and FLR_R primers were designed for PCR on both genomic DNA and cDNA. Due to the high GC content at the 5' end, it was difficult to design forward and reverse primers with a similar melting temperature. In the initial attempts, there was no PCR result even when a gradient PCR was used.

Then the forward PCR primer upstream of the coding region of FLR in genomic sequence was designed for PCR with genomic DNA. The same FLR_R PCR primer was also used here, and the new forward primer and the reverse primer have almost the same melting temperature. New forward PCR primer (FLR_F_bp change) for cDNA was also designed by changing some base pairs but without changing the amino acid identity. The FLR_R primer was also used with this forward primer, and they had very close melting temperatures. PCR experiments were repeated with these primers on both genomic DNA and cDNA, but no PCR products were detected even when different PCR conditions were tested.

Moreover, the primers from the middle of the gene were designed (FLR_F_mid and FLR_R_mid), and they were used with the end primer (FLR_R) and the beginning primer (FLR_F) respectively. In this way, we tried to amplify two parts of DNA for FLR gene, and then to use these PCR products as primers to obtain the full length DNA. But, no PCR products were obtained by using these mid-primers.

Finally, the primers for PCR-mediated mutagenesis were designed. For FLR, the N-terminal non-catalytic domain (first 87 base pairs) was eliminated in order to mimic the active protein kinase. This primer (FLR_F_Mut) was designed from the 90th base pair. PCR was carried out with FLR_F_Mut primer and the FLR_R primer, but no PCR product was detected on the agarose gel.

All of the primers mentioned above are listed in Table 2-2 in Materials and Methods section.

The cloning of FLR was not continued.

2.2.6 Cloning of FLRS

As shown in sections 2.2.3 and 2.2.4, FLRS was activated by leaf rust and FHB challenges, so we were interested in if the transgenic plants overexpressing FLRS have different disease resistant properties compared to wild type wheat plants.

FLRS with 1087 base pairs was amplified by PCR (Figure 2-10). Then FLRS was put into pGEM-T-easy vector (Promega, USA), and the FLRS insert was 1015 base pairs long (Figure 2-11). The ligated vector was used in transformation of JM109 High Efficiency Competent Cells (Promega, USA). Blue/white screening was done by using LB/ampicillin/IPTG/X-Gal plates. White colonies generally contain inserts, so white colonies are chosen to do colony PCR (Figure 2-12) to confirm that FLRS gene has been inserted into T-easy vector. The PCR product of the final plasmid is around 1kb (Figure 2-12), indicating successful insertion of FLRS.

Four repeats of colony PCR were performed, and three of them were sent to Plant Biotechnology Institute, National Research Council, Saskatoon, SK for sequencing. Two of the sequencing samples are exactly same as each other, and the nucleic acid alignment was performed between our FLRS sequencing sample and the FLRS sequence from GenBank (Figure 2-13). The result shows 86.2% identity in 1192 residues overlap between these two sequences, but there is one additional sequence part in our sequencing samples, which is shaded in yellow (Figure 2-13). This additional sequence is translated into amino acid sequence based on the right open reading frame (ORF) of full length FLRS (Figure 2-14). The result shows that there are two stop codons in this additional sequence segment (Figure 2-14).

In order to create FLRS transgenic plants, we tried to ligate FLRS gene into pLRCWHEAT2-gevAgdv vector (Figure 2-15) (Lethbridge Research Centre, Agriculture and Agri-Food Canada). We choose Pst I and XbaI as the two restriction sites (Figure 2-15), which are on the two sides of ccdB gene. Based on our sample sequence ORF, a pair of primers was designed and they included the two restriction sites, PstI and XbaI, respectively. The primer sequences are listed in materials and methods section. FLRS was then amplified by PCR. The PCR product is around 1kb (Figure 2-16). The FLRS PCR product and pLRCWHEAT2-gevAgdv vector were digested with PstI and XbaI restriction enzymes (Figure 2-17). The restriction products of FLRS and vector were ligated together, and the transformation was performed. Colony PCR on FLRS was carried out to confirm the pLRCWHEAT2-gevAgdv-FLRS construct (Figure 2-18). Then, pLRCWHEAT2-gevAgdv-FLRS construct sample will be sent to Lethbridge Research Centre of Agriculture and Agri-Food Canada for transgenic work.

Figure 2-10: PCR amplification of FLRS from wheat. The 1kb ladder is indicated at the left of the gel. Leaves from wheat are collected and the PCR amplification with the corresponding primers is performed on the corresponding cDNA. Three repeat PCR were shown in the agarose gel.

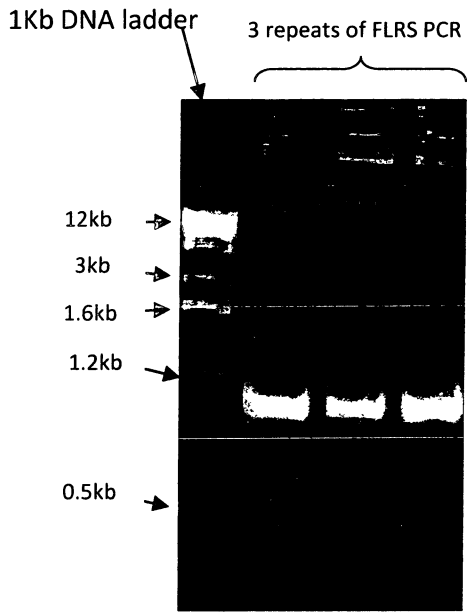


Figure 2-11: pGEM-T Easy vector map.

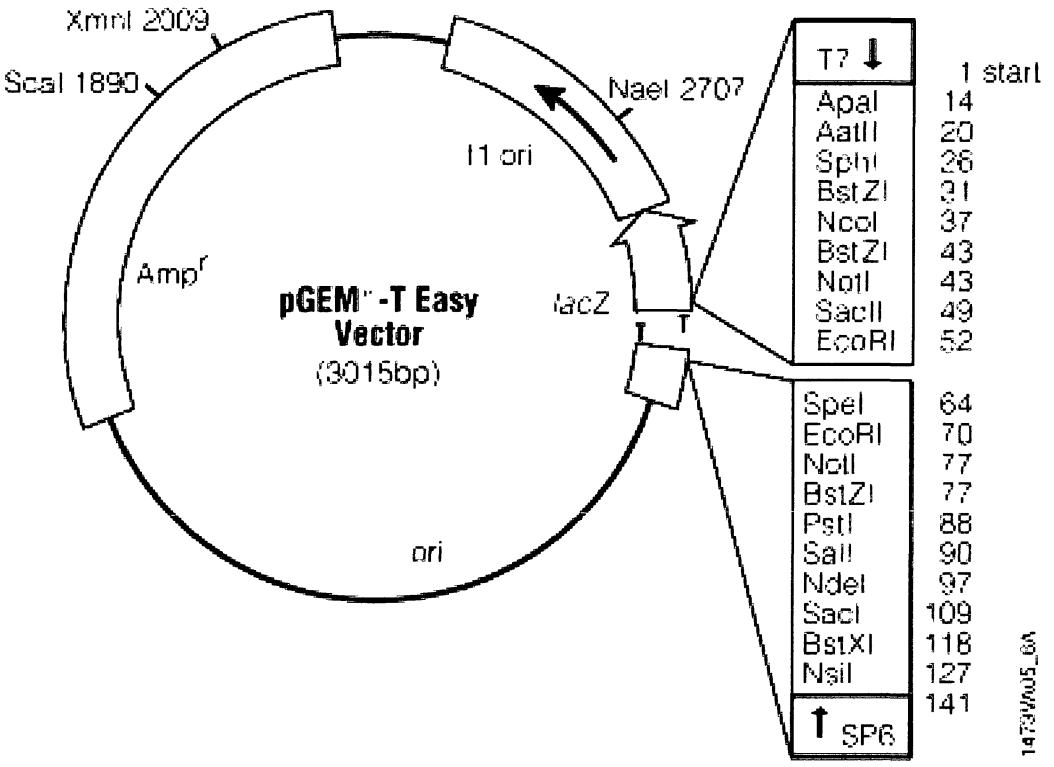


Figure 2-12: Colony PCR for confirmation of T-Easy-FLRS construct. The 1kb ladder is indicated on the left side of the gel. PCR was performed with the same primers as those used for FLRS amplification (Figure 2-10). The four lanes represent the four colonies.

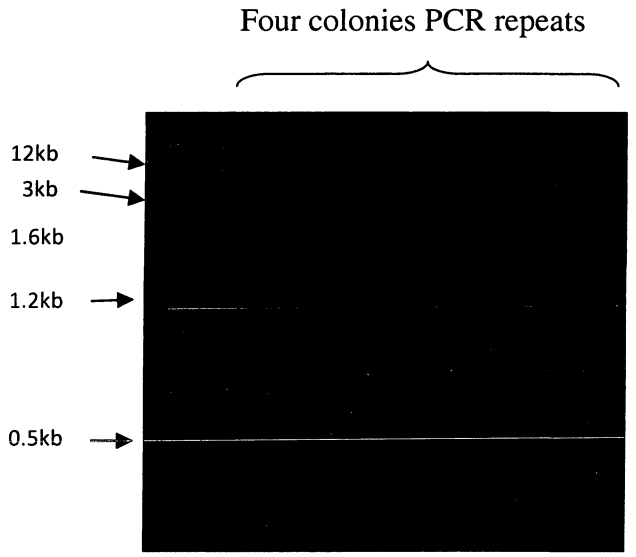


Figure 2-13: Alignment between our PCR amplified FLRS sequence (1) and the one from GenBank (AY173962) (2). 86.2% identity in 1192 residues overlap; Score: 5507.0; Gap frequency: 11.6%. The additional sequence in our PCR product is shaded by yellow.

```
1,            1 ACGTCTTCGAGGTCACCGCCAAGTACAAGCCCCC-ATCCTCCCCATCGGCAAGGGCGCCT
2,          158 ACGTCTTCGAGGTCACCGCCAAGTACAAGCCCCCCATCCTCCCCATCGGCAAGGGCGCCT
                ********************************** ************************

1,           60 ACGGCATCGTCTGCTCCGCGCTCAACTCCGAGACCGGGGAGCAGGTGGCCATCAAGAAGA
2,          218 ACGGCATCGTCTGCTCCGCGCTCAACTCCGAGACGGGGGAGCAGGTGGCCATCAAGAAGA
                ********************************** ************************

1,          120 TCGCCAACGCCTTCGACAACAAGATCGACGCCAAGCGCACGCTGCGGGAGATCAAGCTGC
2,          278 TCGCCAACGCCTTCGACAACAAGATCGACGCCAAGCGCACGCTGCGGGAGATCAAGCTGC
                ************************************************************

1,          180 CCCGCCACATGGACCACGAGAATATTGTTGCAATAAGGGATATTATACCTCCTGCACAGA
2,          338 TCCGCCACATGGACCACGAGAATATTGTTGCAATAAGGGATATTATACCTCCTGCACAAA
                 ************************************************************ *

1,          240 GGACTGCATTCAATGATGTCTATATTGCATATGAATTGATGGACACCGATCTGCATCAAA
2,          398 GGACTGCATTCAATGATGTCTATATTGCATATGAATTGATGGACACCGATCTGCATCAAA
                ************************************************************

1,          300 TTATTCGCTCAAATCAAGCTTTATCGGAGGAGCACTGCCAGTATTTCCTTTATCAGATCC
2,          458 TTATTCGCTCAAATCAAGCTTTATCGGAGGAGCACTGCCAGTATTTCCTTTATCAGATCC
                ************************************************************

1,          360 TTCGTGGCTTGAAGTATATACATTCAGCAAATGTTCTCCACCGAGACTTGAAGCCTAGCA
2,          518 TTCGTGGCTTGAAGTACATACATTCAGCAAATGTTCTCCACCGAGACTTGAAGCCTAGCA
                *************** ********************************************

1,          420 ATCTTCTATTGAATGCAAACTGTAACCTAAAAATTTGTGATTTTGGACTTGCTCGTACCA
2,          578 ATCTTCTTTTGAATGCAAACTGTGACCTAAAAATTTGCGATTTTGGGCTTGCTCGTACCA
                ******* *************** ************* ******** *************

1,          480 CCTCAGAAACTGATTTTATGACTGAGTATGTTGTGACAAGATGGTACAGGGCACCAGAGC
2,          638 CCTCAGAAACTGATTTTATGACCGAGTATGTTGTGACAAGATGGTACAGGGCACCAGAGC
                ********************** ************************************

1,          540 TTCTGTTGAACTCCTCTGAATATACTGCAGCAATTGATGTGTGGTCTGTGGGCTGTATAT
2,          698 TTTTGTTGAACTCCTCCGAATACACTGCAGCAATTGATGTGTGGTCTGTGGGCTGTATAT
                ** ************* ***** ************************************

1,          600 TTATGGAACTGATGGATCGGAAACCTTTGTTTCCGGGAAGGGACCATGTCCATCAGCTAC
2,          758 TTATGGAACTGATGGACCGGAAACCGTTGTTTCCAGGAAGAGACCATGTCCATCAGCTAC
                **************** ******** ******** ***** ******************

1,          660 GCCTACTAATGGAGGTTAGAAAGTACCCTATGAACTTCTTTTACATCGAATTTAAATCAT
2,          818 GTCTACTAATGGAGGTT-------------------------------------------
                * ***************

1,          720 GTTGTGTGATCCTGTTTTAGCATAATAGAGGTATTTAAGCACTTTCTGGTATATATAATA
2,          835 ------------------------------------------------------------


1,          780 AGTACTTTTCTGGTGCACATTAATTCATTTTCAATTCCCTTGTGGTATTTCCTCGCTGCA
2,          834 ---------------------------------TTCCCTTGTGGTATTTCCTCGTTGCA
                                                  ******************** ****
```

```
1,          840 GCTCATTGGAACACCAAATGAGGCCGATTTGGATTTTGTAAATGAAAACGCAAGAAGATA
2,          860 GCTCATTGGAACACCAAATGAGGCCGATTTGGATTTTGTAAATGAAAATGCAAGAAGATA
                ********************************************** ***********

1,          900 TATCCGCCAACTTCCCCGTCATGCAAGGCAATCATTATCTGAGAAGTTTCCACATGTTCA
2,          920 TATCCGCCAACTTCCCCGTCATGCAAGGCAATCATTATCTGAGAAGTTTCCACATGTTCA
                ************************************************************

1,          960 CCCTTCAGCAATTGACTTGGTTGAAAAGATGCTGACTTTCGATCCTAGACAGAGAATAAC
2,          980 CCCTTCAGCAATTGACTTGGTTGAAAAGATGCTGACTTTCGATCCTAGACAGAGAATAAC
                ************************************************************

1,         1020 AGTTGAAGGTGCACTTGCGCATCCTTACTTGGCATCGCTGCATGACATAAGTGATGAGCC
2,         1040 AGTTGAAGGCGCACTTGCGCATCCTTACCTGGCATCGCTGCATGATATAAGTGATGAGCC
                ********* ***************** ********** ********** **********

1,         1080 AGTCTGCACGATGCCCTTCAGCTTTGACTTCGAGCAGCATGCGTTGTTGGAAGAACAAAT
2,         1100 AGTCTGCACGATGCCCTTCAGCTTTGACTTCGAGCAGCATGCGTTGTCGGAAGAACAAAT
                ********************************************** ************

1,         1140 GAAGGATCTAATCCACCAAGAGGGCATCTCGTTCAACCCTGATTACCAGTTG
2,         1160 GAAGGATCTAATCCACCAGGAGGGCATCACGTTCAACCCTGATTACCAGTAG
                ***************** ********* ********************** *
```

Figure 2-14: Translation from additional DNA sequence (from 676th to 814th base pairs in Figure 2-13) to protein sequence.

TTAGAAAGTACCCTATGAACTTCTTTTACATCGAATTTAAATCATGTTGTGTGATCCTGTTTTAGCATAAT

L E S T L **Stop** T S F T S N L N H V V **Stop** S C F S I I

AGAGGTATTTAAGCACTTTCTGGTATATATAATAAGTACTTTTCTGGTGCACATTAATTCATTTTCAA

E V F K H F L V Y I I S T F L V H I N S F S

Figure 2-15: pLRCWHEAT2-gevAgdv vector map. The restriction sites used in our work are shaded in yellow colour.

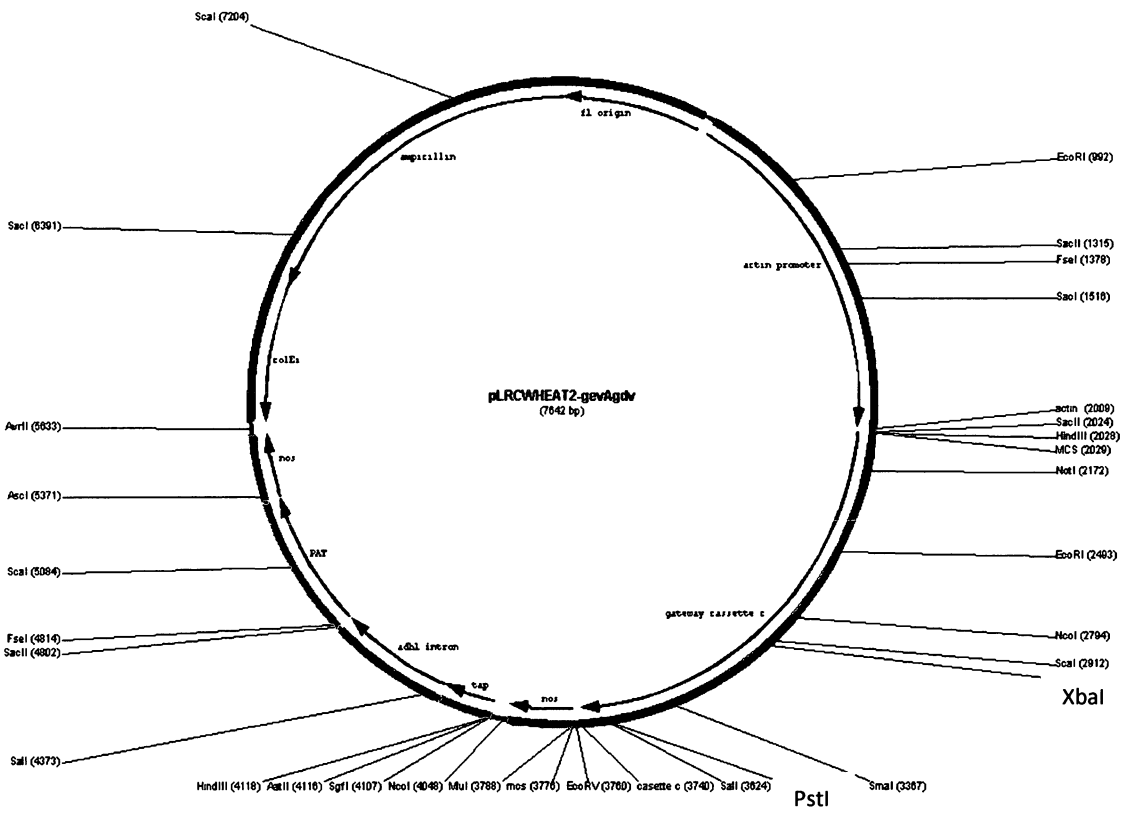


Figure 2-16. PCR amplification of FLRS. The 1kb ladder is indicated at the left of the gel. The PCR amplification with the corresponding primers was performed on our sequencing sample. Two PCR repeat experiments were shown in the agarose gel.

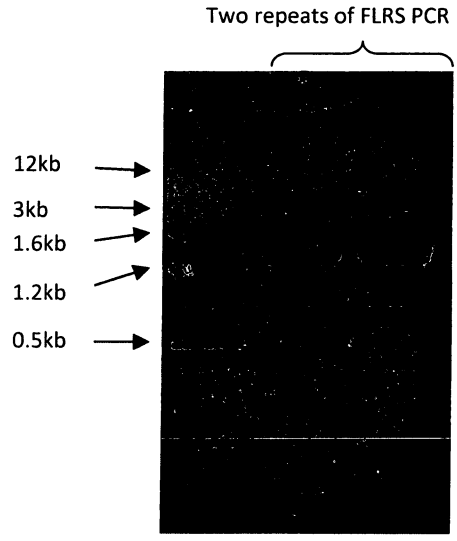


Figure 2-17: Restriction enzyme digestions of FLRS and pLRCWHEAT2-gevAgdv by PstI and XbaI. The 1kb ladder is indicated on the left of the gel. FLRS was cut into multiple pieces by restriction enzymes, and the top bands (indicated by red arrow) were used for ligation. Three repeats of FLRS digestion have been carried out. The last right lane on the gel is the digestion of pLRCWHEAT2-gevAgdv vector, and the ccdB lethal gene (0.6kb) was cut from the vector.

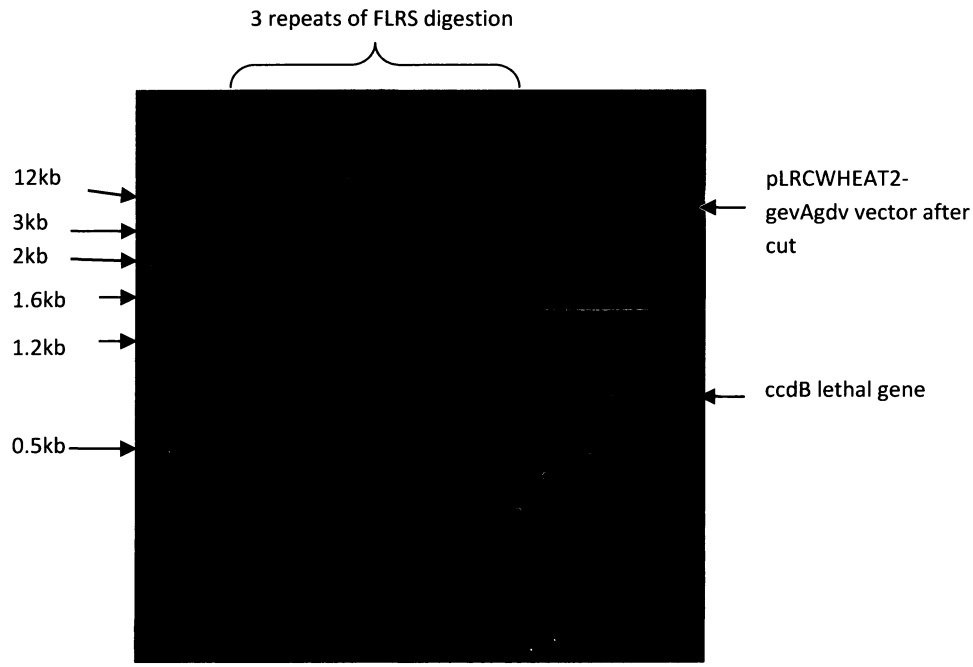
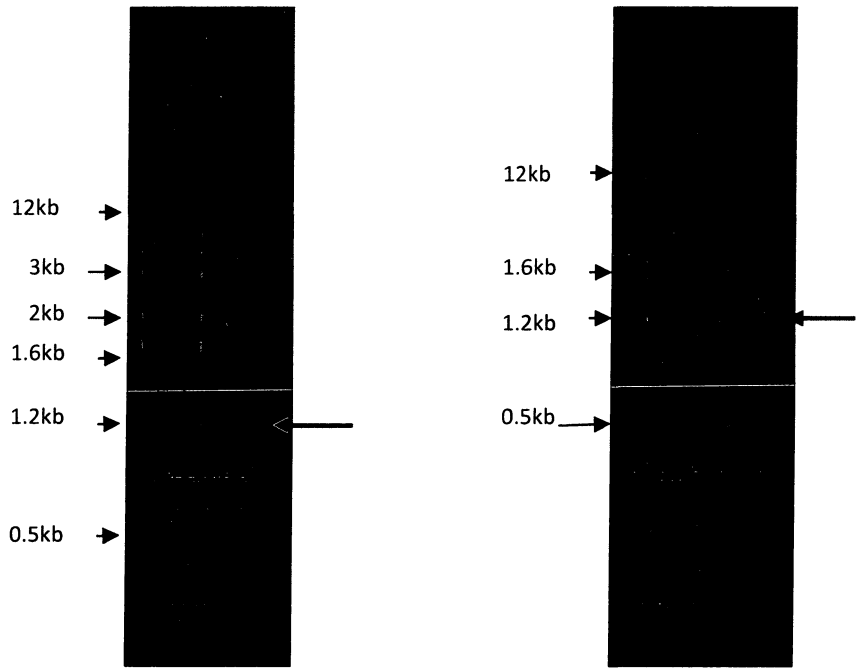


Figure 2-18: Colony PCR for confirmation of pLRCWHEAT2-gevAgdv-FLRS construct. The 1kb ladder is indicated on the left side of the gel. PCR was performed with the same primers used for FLRS amplification (Figure 2-10). The right size band for FLRS gene is indicated by red arrow. Two repeats of colony PCR were performed.



2.3 Discussion

2.3.1 FLR and FLRS are identified as MAPK pathway components

The family of MAPKKKs forms the largest group of MAPK pathway components. *Arabidopsis* contains 80 MAPKKKs (Jonak *et al.,* 2002), which can be subdivided into three major subtypes, Raf, MEKK and ZIK (Rao *et al.,* 2010). 'GTPEFMAPE' is the conserved signature motif of ZIK subfamily (Rao *et al.,* 2010). This conserved signature motif is found in FLR sequence, suggesting FLR belongs to ZIK family. In MAPK cascade, MAPKKs are activated when serine and serine/threonine residues in the S/TXXXXXS/T motif are phosphorylated by MAPKKKs (Ichimura *et al.,* 1998). 30 to 301 amino acids residues from FLR are identified as catalytic domains of protein kinases, which are also identified as the domain of serine/threonine protein kinase. This is an additional evidence to indicate that FLR is a MAPKKK. Moreover, alignment between FLR protein and putative MAPKKK protein from *Homo sapiens* shows certain similarity, and both of them contain ZIK signature motif. Therefore, all of these bioinformatics analysis has suggested that FLR is a MAPKKK in wheat.

MAPKs are activated when both tyrosine and threonine residues in the TXY motif are phosphorylated by MAPKKs (Ichimura *et al.,* 1998). FLRS has one T-X-Y motif, and 62 to 357 amino acids from FLRS protein sequence are identified as the protein kinase domain. FLRS and MAP kinase 6 from *Oryza sativa* have high identity. Our analysis has suggested that FLRS is a wheat MAPK.

2.3.2 No change of FLR and FLRS transcript levels by SA and FB1

In plants, MAPK cascade has been implicated in relaying signal of various plant hormones and stress stimuli (Jonak *et al.*, 1999; Mizoguchi *et al.*, 1997; Nishihama *et al.*, 1995). FLR and FLRS are identified as components of MAPK cascade, so it is possible that these two genes are defense related genes.

Salicylic acid (SA) has been identified as a key signaling component in numerous plant responses to both biotic and abiotic stresses (Gaffney *et al.,* 1993; Glazebrook, 1999; Rao and Davis, 1998; Surplus *et al.,* 1998; Grant and Lamb, 2006). Although it has been suggested that SA is essential for many plant defense responses, under other circumstances SA did not seem to be required. Table 2-1 shows that mRNA levels of FLR and FLRS were not changed after SA treatment, indicating that these two genes may not be involved in SA-mediated pathway.

Fumonisin B1, a PCD-eliciting mycotoxin, has been used in model systems for the study of plant cell death in pathogen response pathways (Gilchrist, 1998). FB1 is used as a biotic stress in this work. The result shows that FB1 did not induce FLR and FLRS changes at mRNA level (Table 2-1), suggesting transcriptional regulation of FLR and FLRS may not play a role in wheat response to FB1.

However, the MAPK cascade components are regulated at multiple levels, which include transcriptional, translational and post-translational levels (Zhang *et al.,* 2000). In a study of a variety of stresses, Ichimura *et al.* (2000) showed that low temperature, low humidity, hyper-osmolarity, touch and wounding induce rapid and transient activation of the *Arabidopsis* MAP kinases ATMPK4 and ATMPK6. Activation of ATMPK4 and ATMPK6 was associated with tyrosine phosphorylation but not with the amounts of mRNA or protein. Also, in the examination of proteins that were phosphorylated in *Arabidopsis* upon treatment with flagellin, a bacterial elicitor, only a few of the phosphoproteins were found to be regulated at the transcriptional level (Peck, 2003). Since transcript levels do not necessarily reflect the amount of final active protein product (Buchanan-Wollaston *et al.,* 2005, Xing *et al.,* 2002), these two kinases will be further analyzed at translational and post-translational levels later in this study. For simplicity, no change on transcript abundance is often referred to as no induced gene expression.

2.3.3 Transcriptional changes of FLR and FLRS genes in response to leaf rust challenge and *Fusarium graminearum* challenge

Although it is shown in section 2.3.2 that FLR and FLRS, as MAPK pathway components, may not play roles in the SA-mediated pathway or in response to FB1 at transcriptional level, we still would like to know if these two genes are involved in plant-pathogen interactions.

Wheat near isogenic line Thatcher Lr16 was inoculated with the leaf rust pathogen, race BBB or race TJB. The activation of plant defense responses is initiated upon plant-pathogen recognition, mediated either by a gene-for-gene interaction between a plant resistance (R) gene and a pathogen avirulence (Avr) gene, or by the binding of a non race-specific elicitor to the receptor (Baker *et al.,* 1997; Dangland Jones, 2001; Hammond-Kosack and Jones, 1996; Martin, 1999). Race BBB, an incompatible leaf rust challenge, has the Avr gene that can be recognized by the Lr16 R gene product, leading to plant defense responses. Race TJB, a compatible leaf rust to Thatcher Lr16, doesn't have a corresponding Avr gene that can be recognized by Lr16, so TJB challenge causes disease symptom in Thatcher Lr16. Both FLR and FLRS are transcriptionally up-regulated upon Lr16-BBB incompatible interaction at 1.5 hours compared to Lr16 control and Lr16-TJB compatible interaction (Figure 2-5 and Figure 2-6), suggesting FLR and FLRS are involved in wheat defense response to leaf rust.

Susceptible and resistant wheat cultivars were challenged by *Fusarium graminearum.* Upon this fusarium head blight challenge, FLR gene was activated in resistant cultivar Frontana (Figure 2-7). In order to find out if there is a correlation between the mRNA level of FLR and resistance or susceptibility, Northern blotting was performed to detect background transcriptional levels of FLR gene in different resistant and susceptible cultivars (Figure 2-8). The result has indicated that there is no clear correlation between FLR transcript level and resistance/susceptibility (Figure 2-8). Therefore, FLR activation in FHB resistant cultivars Frontana suggests that it is the enhanced level (above the background level in each cultivar) that may contribute to the resistance to FHB. FLRS gene was up-regulated in Frontana (resistant cultivar) in response to FHB at 24 hours

(Figure 2-9), but there was no change in Roblin (susceptible cultivar). This may indicate that FLRS is involved in the plant-pathogen interaction in FHB resistant cultivars. Work on other resistant and susceptible cultivars may further evaluate the role of these two kinase genes in wheat-FHB interaction. Further Southern analysis may help indicate whether there are multiple copies of FLR or FLRS genes in the genome. Also, qPCR may provide a more quantitative evaluation.

Many studies showed that MAPK pathway plays a role in defense response to pathogen attacks. MPK6-silenced *Arabidopsis* plants are compromised in resistance to different pathogens (Menke *et al.*, 2004). Dóczi *et al.* (2007) revealed that the *Arabidopsis* MKK3 pathway plays a role in pathogen defense. Also, Marcel *et al.* (2007) showed that mitogen-activated protein kinase kinase kinase (MAP3K) in barley contributes to partial resistance to barley leaf rust. Our recent study also showed that the mitogen-activated protein kinase kinase (MAPKK) tMEK2 from tomato (*Solanum lycopersicum*) is a key component in the defense pathways against pathogen attacks, because tMEK2-transgenic wheat (*Triticum aestivum*) has partial resistance to wheat leaf rust (caused by *Puccinia triticina*) (Fan *et al.,* 2009).

2.3.4 Cloning of FLR and FLRS

In this chapter, FLR and FLRS are identified as MAPK pathway components based on bioinformatics analysis. Our experimental data suggests that FLR and FLRS are not involved either in the SA mediated pathway or in response to FB1 at transcriptional level. However, our results have indicated FLR and FLRS are involved in defense responses to leaf rust and Fusarium head blight challenge in wheat. Since they are involved in plant-pathogen interactions, we tried to clone these two genes and to make transgenic plants with overexpression of FLR and FLRS. Then the phenotypes can be compared between the transgenic plants and wild type wheat plants upon leaf rust and FHB challenge. Unfortunately, the cloning work of FLR cannot be continued because the PCR

amplification of FLR did not work. FLRS cloning work will be continued by another student.

Figure 2-14 showed the additional sequence from my sequencing sample includes many stop codons. It suggested that this additional sequence might be an intron. There might be two reasons causing this intron in my sequence sample. First possible reason is that the intron was not successfully spliced during RNA modification after transcription. Second, after RNA extraction and before cDNA synthesis, DNase I was used to eliminate the extra DNA from RNA sample. The technique problem might hinder the proper function of DNase I so additional DNA was left in the RNA sample.

2.4 Material and methods

2.4.1 Bioinformatics analysis

The catalytic domains of FLR and FLRS proteins were searched by querying protein sequences against InterProScan (http://www.ebi.ac.uk/Tools/InterProScan/). The similar sequences of FLR and FLRS were searched against the non-redundant protein database (National Center for Biotechnology Information, NIH, Bethesda) using BLASTP (Altschu SF *et al.,* 1997). Alignments of the selected sequences were done using ExPASy SIM-Alignment tool (Swiss Institute of Bioinformatics) (http://ca.expasy.org/tools/sim-prot.html).

2.4.2 Plant materials and growth conditions

Frontana and Roblin seeds were originally obtained from Cereal Research Centre, Agriculture and Agri-Food Canada, Winnipeg, MB, Canada. Seeds were surface sterilized for 2 min in 70% ethanol and then soaked for 8 min in sterilization solution (25% Bleach v/v and 0.01% Triton X-100 v/v). Seeds were then rinsed 10 times with autoclaved water. After sterilization, seeds were sowed directly to autoclaved soil (BM1, Montreal, Quebec, Canada). Each pot of wheat was fertilized with 7-9 mL granular

14:14:14 slow release fertilizer. They were grown in 16 h of light at 17°C and 8 h of dark at 15°C in growth chambers (ENCONAIR Technologies Inc, Winnipeg, Manitoba).

2.4.3 SA or FB1 treatment

To investigate the transcriptional levels of ERK-type MAPKs in wheat leaves with SA and FB1 treatment, Frontana and Roblin leaves were collected and treated with SA and FB1 for indicated period of times.

Three to four leaves harvested from the 3-week-old plants were cut to ~2cm segments and incubated in 100μM SA (or 5μM FB1) solution under the same growth conditions after infiltration. Leaf segments were placed upward on the filter paper in Petri dishes so that they were just covered by the solution. Leaf segments treated in the same way with an equal volume of distilled water served as controls. Samples after 24 h, 48 h and 72 h incubation were collected. All of the leaf segments were collected in Falcon tubes and snap-frozen in liquid nitrogen. They were stored at -80°C and used for RNA extraction within 3 months.

2.4.4 RNA extraction and RT-PCR

Total RNA was extracted from wheat leaves using TRIzol Reagent kit (Invitrogen, USA) according to manufacturer's protocol. Leaf tissue (100 mg) was homogenized in 1 mL of TRIzol. The homogenized sample was incubated for 5 min at room temperature (RT) to permit the complete dissociation of nucleoprotein complexes. An aliquot of 200μL of chloroform was then added to the sample, mixed vigorously by hand for 15 seconds, and incubated at RT for 2-3 min. The sample was centrifuged for 15 min at 12,000 x *g* at 4°C. Following centrifugation, the mixture separated into a lower, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase. The volume of the aqueous phase was about 60% of the volume of TRIzol reagent used for homogenization. The aqueous phase was transferred

into a fresh tube and 500 µL of isopropyl alcohol was added. The sample was incubated for 10 min at RT and centrifuged for 10 min at 12,000 x *g* at 4°C. The RNA precipitate formed a gel-like pellet on the side and bottom of the tube. The supernatant was removed and the RNA pellet was washed once with 1 mL of 75% ethanol (diluted with DEPC water). The sample was mixed by vortexing and centrifuged 5 min at 7,500 x *g* at 4°C. At the end of the procedure, the RNA pellet was air-dried for 5 min. The pellet was dissolved in 50 µL of RNase-free water and stored at -20°C. RNA was measured using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, USA).

After TRIzol extraction, Deoxyribonuclease I kit (amplification grade, Invitrogen, USA) was used to eliminate genomic DNA contamination in the sample. The following was added to an RNase-free, 0.5 mL microcentrifuge tube on ice: 1-5 µg RNA sample, 1 µL 10X DNase I reaction buffer (100mM Tris-HCL (pH7.5), 25mM $MgCl_2$, 5mM $CaCl_2$), 1 µL of 1U/µL DNase I, and DEPC-treated water to 10 µL. The sample was then incubated for 15 min at RT. DNase I was inactivated by addition of 1 µL of 25 mM EDTA solution to the reaction mixture. The sample was heated for 10 min at 65°C.

Next, the Cloned AMV First-Strand cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA) was used for cDNA synthesis according to the manufacturer's protocol. A master mixture of 4 µL of 5x cDNA synthesis buffer (250 mM Tris acetate (pH8.4), 375 mM potassium acetate, 40 mM magnesium acetate, stabilizer, 20 µg/mL BSA), 1 µL of 0.1 M DDT, 1 µL of RNaseOUT (40U/µL), 1.5 µL of DEPC-treated water and 0.5 µL of cloned AMV RT (15U/µL) was prepared and kept on ice. Also, in a 0.5 mL tube, the following components were mixed: 1 µL of 50 µM $Oligo(dT)_{20}$, 1-5 µg RNA sample, 2 µL of 10 mM dNTP mix and DEPC-treated water to 12 µL. The mixture was incubated for 5 min at 65°C. Eight µL of the master mixture was added to the above 0.5 mL reaction tube. Then, the reaction tube was transferred to a thermal cycle and heated for 48 min at 48°C, and then for 5 min at 85 °C.

The primers used for RT-PCR were 5'-GGCCATTCGAGGAACATCTA-3' and 5'-TTGTCGTCATTATCGGCGTA-3' for FLR, and 5'-CAGGTGGCCATCAAGAAGAT-3' and 5'-GCAGTGCTCCTCCGATAAAG-3' for FLRS. A wheat acting gene (GenBank

accession number AB181991) with the primer sequences 5'-GCCACACTGTTCCAATCTATGA-3' and 5'-TGATGGAATTGTATGTCGCTTC-3' was used as an internal control. RT-PCR was carried out under the following conditions: 94 °C for 1 min; 1min at 94°C, 1min at 61°C, and 1min at 72°C for 25 cycles; and then 10 min at 72°C. Sizes of FLR and FLRS PCR product are 218 bp and 237 bp respectively. Size for the wheat actin gene PCR product is 369 bp long.

2.4.5 Leaf rust challenge and *Fusarium graminearum* challenge on wheat, and Northern blotting

Plant materials and leaf rust inoculation - Wheat seeds (from Cereal Research Centre, Agriculture and Agri-Food Canada, Winnipeg, MB, Canada) were grown in the greenhouse with supplemented lighting using high pressure sodium light bulbs, 16 h daylight, 18-22°C. Nine-day old plants were inoculated with wheat leaf rust *Puccinia recondita* race BBB (avirulent) and race TJB (virulent), suspended in a light mineral oil, Bayol (Esso, Canada). The leaf rust culture was originally derived from a single spore isolate, which was increased, vacuum dried and stored at 4°C.

Plant materials and FHB inoculation - Wheat seeds (from Cereal Research Centre, Agriculture and Agri-Food Canada, Winnipeg, MB, Canada) were planted in plastic cones with Metro Mix, one seed per cone, 54 plants per wheat line. Plants were grown in a controlled environment with a 16 h photoperiod and 15/13°C day/night temperature regime to promote tillering. The cones were placed in trays of water then transferred to Nutri-Bloom hydroponic solution after seedling emergence. Leaf tissue was collected from the lower leaves and stored at -80°C for gene expression studies. The wheat spikes were inoculated at anthesis (7-8 weeks after germination).

Isolate EEI 20/6 of *Fusarium graminearum* was used as the inoculum source. A standard conidial spore suspension was prepared in a carboxymethyl cellulose media. Spore concentration was adjusted to 50,000 conidia/μL. A spray bottle was used to apply 3-5mL of spore suspension to wheat spikes. The control plants were treated with water

and Tween 20. The plants were placed in a humidity chamber equipped with a timer set to shut off after 6 hours. The plants remained in the chamber 18 hours post inoculation. Percent infection was determined 21 days post inoculation based on the number of infected spikelets compared to the total number.

Northern blotting - Fifteen mg of RNA per lane was separated on a denaturing formaldehyde gel. Following transfer to nylon membranes, the blot was hybridized with radiolabelled fragment of FLR and FLRS coding regions and hybridization signals were visualized by autoradiography (Sambrook *et al.*, 1989).

2.4.6 Genomic DNA extraction from wheat

Genomic DNA was extracted from 3-week-old wheat (Fielder cultivar) by Extract-N-Amp^{TM} Plant PCR kit (Sigma, USA). 100 μL of the Extraction Solution was added to the collection tube, which was then closed and vortexed briefly. After incubation at 95°C for 10 minutes, 100 μL of the Dilution Solution was added and the samples were vortexed to mix. The diluted samples were stored at 2-8°C for PCR use.

2.4.7 FLR amplification by PCR

I tried to amplify FLR using three sets of PCR reactions. Total DNA was isolated from 3-week-old seedlings using Sigma Extract-N-Amp^{TM} Plant PCR kit following the manufacturer's instruction. Five separate PCR amplifications were carried out to obtain the FLR gene. The reaction mixtures were performed as 1 min at 94°C, followed by 1min at 94°C, 1 min at 55°C to 65°C (depending on the primers' melting temperatures), 1min 30s at 72°C for 34 cycles, and then 72°C for 10min. All of the five sets of primers are listed in Table 2-2. The first pair of primers, FLR_F and FLR_R, was designed from the beginning and the end of FLR open reading frame (ORF). The second pair of primers, FLR_F_gDNA and FLR_R, is designed to amplify genomic DNA. FLR_F_gDNA was designed from the upstream of the coding region. To amplify the full length cDNA, the

third pair of primers, FLR_F_bp_change and FLR_R, was used. New forward PCR primer (FLR_F_bp change) was designed by changing five base pairs (underlined in Table 2-2) but keeping the same amino acids identity. The fourth set of primers is FLR_F_mid with FLR_R, and FLR_F with FLR_R_mid. FLR_F_mid and FLR_R_mid were designed from the middle part of FLR gene. Two parts of FLR gene were amplified, and then these PCR products were used as primers to obtain the full length DNA. The last set of primers is FLR_F_Mut and FLR_R. The N-terminal non-catalytic domain (first 87 base pairs) was eliminated in order to mimic the active protein kinase. FLR_F_Mut was designed from the 90th base pairs of FLR gene.

Table 2-2: PCR primers for FLR cloning. F: forward; and R: reverse; gDNA: genomic DNA; bp: base pair.

Primer name	**Primer sequence**
FLR _F	5'- TTT ATA GGA TCC ATG TCG AGC CCG CGG CGG– 3'
FLR _R	5'- TTT ATA GGA TCC TCA GAA CAT GAA GAA CTG GTG CTT GTC C- 3'
FLR_F_g DNA	5'- TTT ATA GGA TCC CCT CCT CTC TGG CGT CTT CTG –3'
FLR_F_bp_change	5'- TTT ATA GGA TCC ATG TCG AGT CCA CGT CGT CCA –3'
FLR_F_mid	5'- TAT TGG GCC TCC TCC GAC TCG –3'
FLR_R_mid	5'- TCA AGT CGA GTC GGA GGA GGC CCA ATA-3'
FLR_F_Mut	5'- TTT ATA GGA TCC GGC CGG TTC GAC GAG CTC–3'

2.4.8 FLRS cloning

I tried to amplify FLRs using three sets of PCR reaction. RNA was extracted from 3-week-old wheat leaves as described previously and cDNA was synthesized using Cloned AMV First-Strand cDNA Synthesis Kit (Invitrogen, USA) following the manufacturer's instruction. PCR was carried out to amplify FLRS gene. The PCR amplification protocol was as follows: 1 min at 94°C, followed by 1 min at 94°C, 1 min at 65°C, 1 min 30s at 72°C for 34 cycles, and then 72°C for 10 min. Forward primer 5'- ATG GGA CCC ATC CTC CCC AT -3' and reverse primer 5'- CTA CTG GTA ATC AGG GTT GAA CGA GAT GCC C -3' were used to amplify FLRS gene, which was then ligated to the T-Easy vector following the manufacturer's instruction (Promega, USA), resulting in the plasmid T-Easy-FLRS. The ligated vector was transformed into JM109 High Efficiency Competent Cells following the manufacturer's instruction (Promega, USA). Positive clones were identified from white/blue screen, and verified by colony PCR with the same primers. T-Easy-FLRS from competent cells was extracted and purified using Wizard Plus Minipreps DNA purification kit (Promega, USA) following the manufacturer's protocol. The resulting plasmid was sent to Plant Biotechnology Insttitute, National Research Council, Saskatoon, SK for sequence confirmation. This sequence and the GenBand sequence of FLRS were compared using ExPASy SIM-Alignment tool (Swiss Institute of Bioinformatics) (http://ca.expasy.org/tools/sim-prot.html).

pLRCWHEAT2-gevAgdv vector was recovered from CloneSaver Card (Whatman BioScience, USA) following the manufacturer's protocol.. The FLRS was amplified from T-Easy-FLRS by PCR with primers 5'-GCC CTG CAG CTA CTG GTA ATC AGG GTT GAA CGA GAT GCC C-3' and 5'-TTA TCT AGA ACC ATG GGA CCC ATC CTC CCC ATC GGC AA-3' and the PCR products and pLRCWHEAT2-gevAgdv were digested by PstI and XbaI. Then FLRS was inserted into the corresponding site of the construct to generate pLRCWHEAT2-gevAgdv-FLRS, which was then overproduced in *E.coli*. Plasmid DNA from *E.coli* cells was extracted and purified using Wizard Plus Minipreps DNA purification kit following the manufacturer's protocol. The resulting plasmid will be sent to Lethbridge Research Centre of Agricutlure and Agri-Food Canada to produce FLRS transgenic plants.

Chapter III

Post-translational Activation of ERK-type MAPK in Wheat during Biotic Stresses

3.1 Introduction

Protein phosphorylation plays a crucial role in activating and coordinating pathways in plants that sense and respond to environmental stresses and pathogen attacks. MAPK pathway is identified as one of the key phosphorylation pathways. The cascade components are regulated at multiple levels, such as transcriptional, translational and post-translational levels (Xing *et al.,* 2002). Many studies have described the posttranslational activation of MAPKs homologous to *Arabidopsis* AtMPK6/tobacco (*Nicotiana tabacum*) SIPK (herein referred to as MPK6 homologs belonging to the A2 subgroup; Ichimura *et al.,* 2002) and *Arabidopsis* AtMPK3/tobacco WIPK (MPK3 homologs belonging to the A1 class subgroup). The simultaneous posttranslational activation of MPK6 and MPK3 homologs has been reported during both Avr-R-mediated disease resistance reactions (Zhang and Klessig, 1998a; Romeis *et al.*, 1999; Jin *et al.*, 2003; Pedley and Martin, 2004; Stulemeijer *et al.,* 2007).

In Chapter II, our results demonstrated that the transcriptional levels of FLR and FLRS were not changed under SA and FB1 treatments, but Buchanan-Wollaston *et al.* (2005) have suggested that transcript levels do not necessarily reflect the amount of final active protein product. An interesting observation is that mRNA level of FLRS in Roblin is constitutively higher even before FHB challenge. It would be interesting to know if the gene activity is also regulated at post-translational levcl. Previous studies have indicated that the final effectiveness of protein kinases is regulated at multiple levels, and one of the key regulatory mechanisms is protein phosphorylation (Xing *et al.*, 2002). Rudd *et al.* (2008) used an anti-active MAPK antibody (anti-ERK-^{P}TEY^{P}) on western blots to demonstrate that the wheat mitogen-activated protein kinases TaMPK3 and TaMPK6 were posttranslationally activated during pathogen attacks in wheat.

On the basis of their signature activation sequences, MAPKs can be categorized into at least three broad subfamilies: c-Jun NH_2-terminal kinases (JNKs), p38 MAPKs, and ERKs (Schramek, 2002). To achieve activation, JNKs undergo dual phosphorylation at their Thr-Pro-Tyr motif. The p38 MAPK family are primarily activated at dual phosphorylation sites in their Thr-Gly-Tyr motif. ERKs, on the other hand, are activated by dual phosphorylation of a C motif (Schramek, 2002). FLRS gene is identified as an ERK-type MAPK, since it contains Thr-Glu-Tyr motif. In our study, we used this available anti-active MAPK antibody to prove that extracellular signal-regulated kinase (ERK)-type MAPKs (include FLRS) become posttranslationally activated during treatment with SA and FB1 in both FHB resistant and susceptible cultivars. This result led us to test the activation on the ERK pathway downstream defense responses, such as SAR and PR genes.

SAR is a secondary consequence of defense response, which can be elicited by exogenous treatments with chemical inducers, such as SA, 2, 6-dichloro isonicotinic acid (INA), or benzo (1, 2, 3) thiadiazole-7-carbothioic acid S-methyl ester (BTH) (Ward *et al.* 1991; Uknes *et al.* 1992; Vernooij *et al.* 1995; Friedrich *et al.* 1996; Lawton *et al.* 1996). SAR also can be activated by necrotic lesions, whether these are formed as part of the hypersensitive response (HR) in an incompatible interaction or as a symptom of disease in a compatible interaction (Ryals *et al.* 1996).

It has been known that the induction of specific PR-proteins in dicotyledonous species correlates with the onset of SAR (Ryals *et al.,* 1996; Uknes *et al.,* 1992) and can be induced by exogenous treatments of SA and other related chemical inducers (Friedrich *et al.,* 1996; Vernooij *et al.,* 1995), so increased PR gene expression is often used as a marker for SAR onset. Although SAR remains to be demonstrated in monocots, pathogen and chemical induction of systemic defense responses have been identified (Görlach *et al.,* 1996; Molina *et al.,* 1999; Morris *et al.,* 1998; Schaffrath *et al.,* 1997). Interestingly, Molina *et al.* (1999) showed that PR-1.1 and PR-1.2, considered as indicators of SAR in dicotyledonous plants, were unresponsive to SA and other SAR activators in wheat, a monocotyledonous species, but Lu *et al.* (2006) demonstrated that PR1.2 responded to SA whereas PR1.1 did not. While these results seem contradictory, the responsiveness

could be age- and developmental stage-dependent (Lu *et al.,* 2006). In this chapter, we have examined whether wheat PR1.2 gene responds to SA, which is one of the activators of SAR. In order to know if SAR and PR1.2 are the downstream of ERK-type MAPK, we used ERK docking domain inhibitor and a general kinase inhibitor together with SA treatment.

3.2 Results

3.2.1 ERK-Like MAPKs are post-translationally regulated during SA and FB1 treatments

Protein extracts from SA and FB1 treated wheat levels were subjected to western blot analysis using phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibody (anti-ERK-^{P}TEY^{P}) that specifically detects the dual phosphorylation on Thr and Tyr residues that is an essential feature of their posttranslational activation. The molecular weight of ERK is 42 or 44 kDa. After SDS-PAGE the gel was stained with Coomassie blue. The intensity of the pre-existing protein band at 44kD region was normalized against the intensity of a reference protein at 60kD (large subunit of Rubisco) (Rudd *et al.*, 2008) (Figure 3-1). The pre-existing protein level was not changed after normalization. Then the ratio of the band intensity on western blot and this normalized number is plotted into histogram graph for each case (Figure 3-1) to indicate if there is a change of the cross-reacting protein at phosphorylation level.

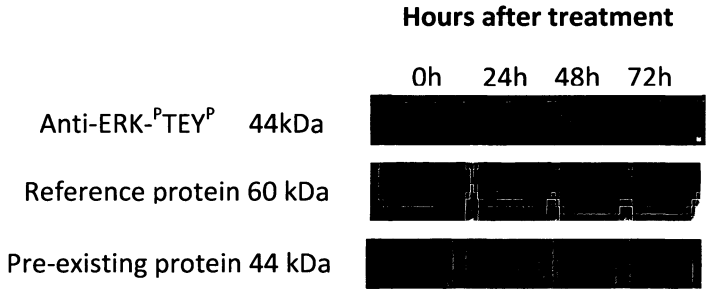
Upon SA treatment for 0 to 72 hours, p44/p42 MAPK became more phosphorylated in resistant cultivar Frontana (Figure 3-1-c), but became less phosphorylated in susceptible cultivar Roblin (Figure 3-1-d). Upon FB1 treatment, the phosphorylation level was enhanced in Frontana (Figure 3-1-a) but no significant change in Roblin from 0 hour to 72 hours (Figure 3-1-b). Graph scales are different, and comparison is made within each cultivar. Two repeats of experiments have been carried out, and the two sets of data are consistent with each other.

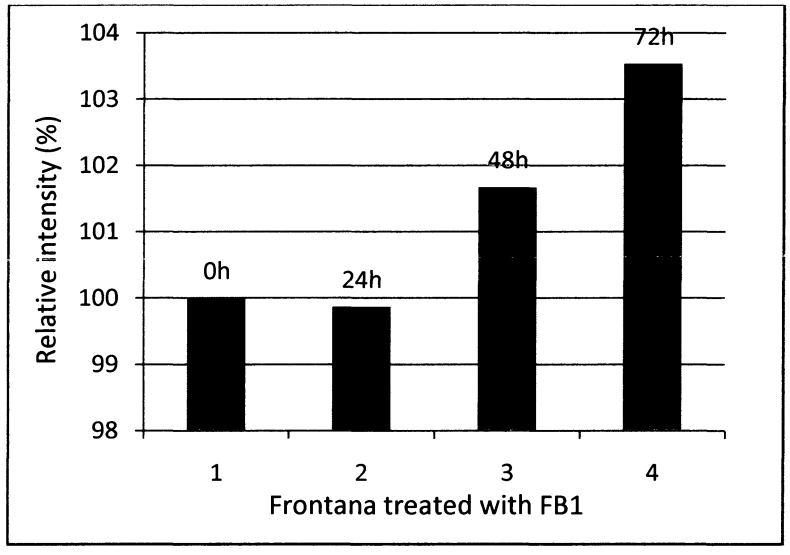
Figure 3-1: ERK-type MAPKs are post-translationally regulated during SA and FB1 treatments in wheat. This is one of the two experiments with similar results.

Western blotting was used for analysis of the activation of ERK-type MAPKs. Protein extracts from leaves treated with SA and FB1 were probed with an anti-active ERK antiserum that recognizes the dual phosphorylated motif present in the active form ($^{P}TEY^{P}$) of ERK-type MAPKs. The 60 kDa region in Coomassie blue-stained gel is shown in each sample and is used for the calculation of the relative amount of protein loadings. The 44-kDa region is indicated in each case for the detectable amount of the pre-existing protein. The band intensity at 44kD region on Coomassie blue-stained gel was normalized against the intensity of the reference protein. Then the ratio of the band intensity on western blot and this normalized number is plotted into histogram graph to obtain relative level of phosphorylation in each sample. Note that graph scales are different, and comparison is made within each cultivar.

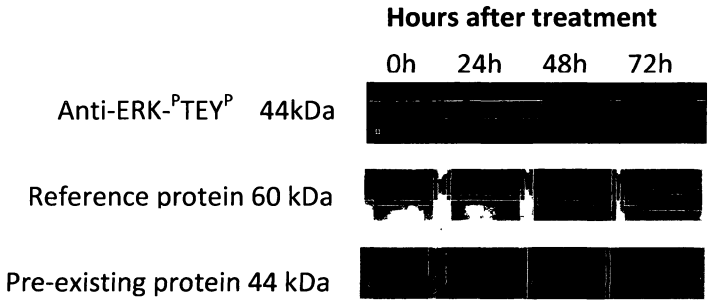
a) Phosphorylation levels of ERK-type MAPK in Frontana during FB1 treatment
b) Phosphorylation levels of ERK-type MAPK in Roblin during FB1 treatment
c) Phosphorylation levels of ERK-type MAPK in Frontana during SA treatment
d) Phosphorylation levels of ERK-type MAPK in Roblin during SA treatment

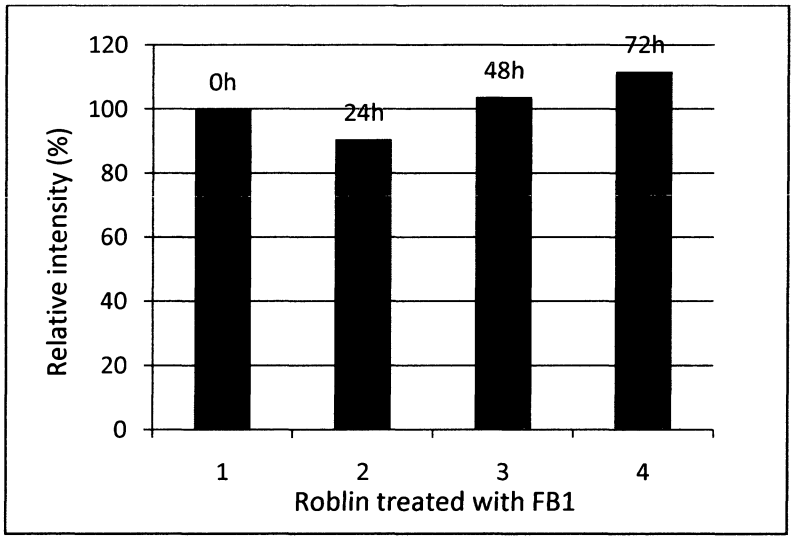
a) Frontana, FB1



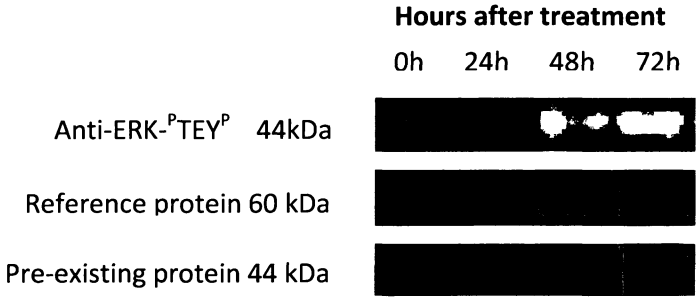


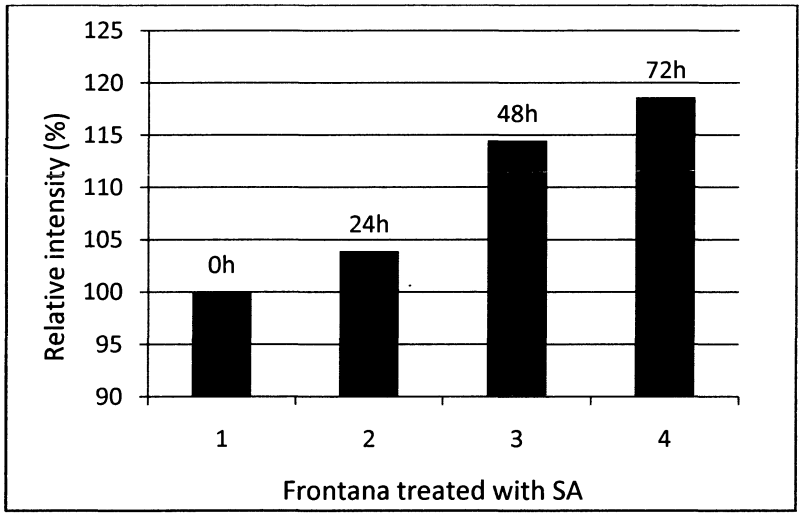
b) Roblin, FB1


Hours after treatment
0h 24h 48h 72h
Anti-ERK-PTEYP 44kDa
Reference protein 60 kDa
Pre-existing protein 44 kDa

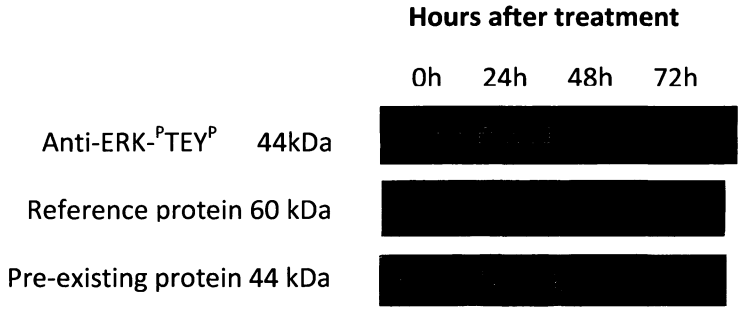

120
100
80
60
40
20
0
Relative intensity (%)
0h
24h
48h
72h
1
2
3
4
Roblin treated with FB1

c) Frontana, SA





d) Roblin, SA


Hours after treatment
0h 24h 48h 72h
Anti-ERK-PTEYP 44kDa
Reference protein 60 kDa
Pre-existing protein 44 kDa


120
100
80
60
40
20
0
Relative intensity (%)
0h
24h
48h
72h
1
2
3
4
Roblin treated with SA

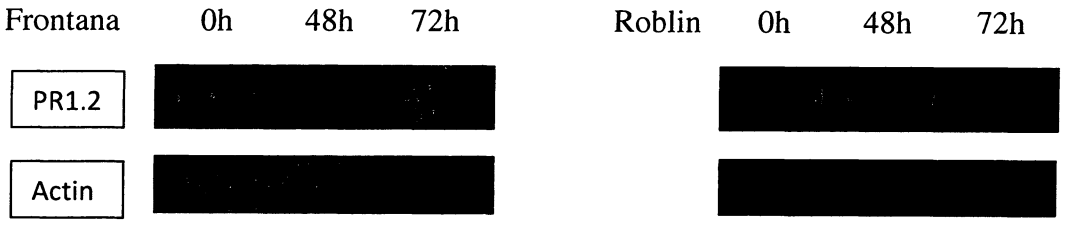
3.3.2 Effect of SA and protein kinase inhibitors on the expression of PR1.2

The expression patterns of PR1.2 gene under SA and water treatments are same in both Frontana and Roblin. From 0 hour to 72 hours, there was no change of the PR1.2 at transcriptional level (Figure 3-2-a and b). The expression levels of actin gene (used as a control gene to ensure equal loading of RNA) under each treatment at different time points were the same (Figure 3-2-a to d). ERK docking domain inhibitor did not reduce the PR1.2 transcriptional level (Figure 3-2-c), whereas PR 1.2 gene expression was decreased by general kinase inhibitor (K252b) at 48 hours and 72 hours (Figure 3-2-d).

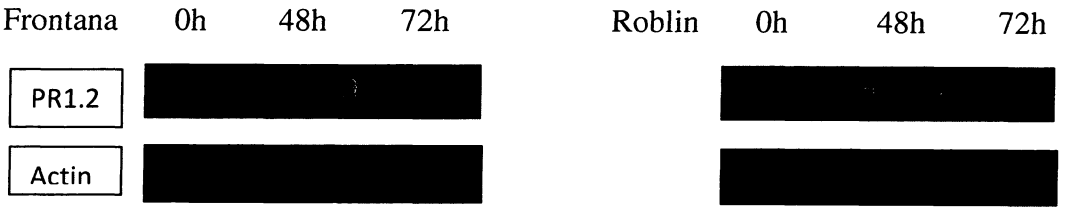
Figure 3-2: Effect of ERK docking domain inhibitor and general protein kinase inhibitor K252b on the expression of PR 1.2 gene (GenBank accession number AJ007349). RNA extracted from leaves of wheat plants (Frontana and Roblin) treated with water, SA and SA plus inhibitors for 0h, 48h and 72h was used in RT-PCR of PR1.2 gene. Wheat actin gene (GenBank accession number: AB181991) was used as the internal standard. Only one experiment was carried out.

a) PR1.2 gene expression in Frontana and Roblin treated with water
b) PR1.2 gene expression in Frontana and Roblin treated with SA
c) PR1.2 gene expression in Frontana and Roblin treated with SA plus ERK docking domain inhibitor
d) PR1.2 gene expression in Frontana and Roblin with SA plus K252b

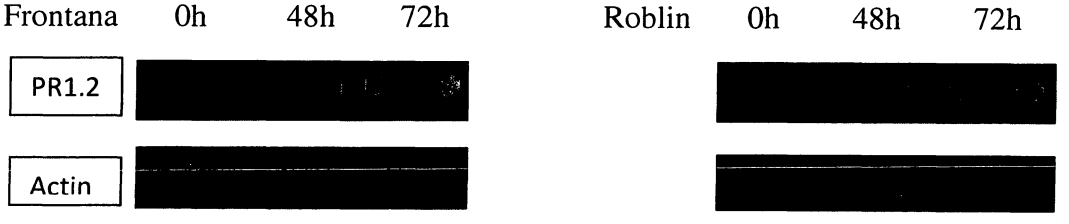
a) Water



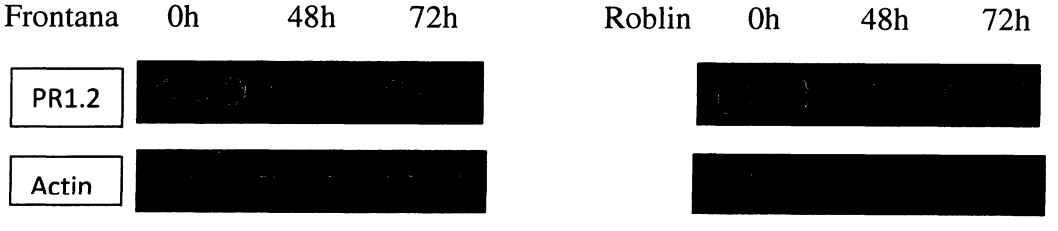
b) SA



c) SA + ERK inhibitor



d) SA+K252b



3.3 Discussion

3.3.1 Post-translational regulation of ERK-type MAPK in wheat during biotic stresses

In order to prevent pathogen replication and/or movement, disease resistance in plants is usually associated with the activation of a wide variety of defense responses. Some rapid responses activated after host plant recognition of an Avr protein or a non-host specific elicitor include the cross-linking of cell wall proteins, the activation of protein kinases, and the increased expression of various plant protectant and defense genes (Hammond-Kosack and Jones, 1996; Yang *et al.*, 1997). It has been known that protein phosphorylation and/or dephosphorylation play essential roles in many signal transduction pathways. Posttranslational modifications can activate an array of defense mechanisms in terms of minutes (Xing *et al.*, 2002; Thurston *et al.*, 2005). Conrath *et al.* (1997) indicated at least two phosphoproteins were implicated in the SA signaling pathway in tobacco, which are members of MAP kinase family SIPK and WIPK. During our gene expression study, ERK-type MAPKs (include FLRS) were subsequently posttranslationally activated by dual phosphorylation of threonine and tyrosine residues in Frontana cultivar during SA and FB1 treatments. It suggests that ERK-type MAPKs in wheat resistant cultivar could be involved in the SA signaling pathway. Also, it indicates that ERK-type MAPKs play a role in wheat defense against FB1 toxin at post-translational level. These results are similar to the observation that SIPK was regulated strictly at the posttranslational level by dual phosphorylation of threonine and tyrosine residues in the conserved TEY motif (Zhang and Klessig, 1997). Our result has also indicated that ERK-type MAPKs were not post-translationally activated in Roblin during SA and FB1 treatments, suggesting ERK-type MAPKs may not be involved in the response to FB1 in Roblin and may not be play a role in SA signaling pathway in this wheat cultivar.

3.3.2 Wheat PR1.2 gene is unresponsive to SA

Molina *et al.* (1999) showed that PR1.2, considered as indicator of SAR in dicotyledonous plants, was unresponsive to SA and other SAR activators in wheat, a monocotyledonous species, but Lu *et al.* (2006) has demonstrated that PR1.2 responded to SA. Interestingly, our results indicated that PR1.2 gene did not response to the activator of SAR, SA, which is consisted with the results previously published for wheat by Molina *et al.* (1999), but contrary to the observations in Lu *et al.* (2006). These differences may be a reflection of varietal differences, since we used wheat cv. Frontana and Roblin for our RT-PCR experiment, and Molina *et al.* (1999) used wheat cv. Kanzler. Lu *et al.* (2006) treated the spring wheat cv. Laura. During our experiment, the SA concentration in our work (100μM) was much lower than the one employed in Lu *et al.* (2006) (20mM) and in Molina *et al.* (1999) (3mM).

An association of PR-1 and the onset of SAR has been reported for *Arabidopsis* and other model dicots (Friedrich *et al.,* 1996; Ryals *et al.,* 1996; Uknes *et al.,* 1992; Vernooij *et al.,* 1995). Genes that respond to SA tend to be acidic PR-proteins (Bart *et al.,* 1998; Ding *et al.,* 2002; Samac *et al.,* 1990; van Loon & van Strien, 1999) and their transcripts frequently possess signal peptides that direct secretion of PR proteins into intercellular spaces. These results are differing from our results on monocot plant wheat. There may be inherent differences between monocots and dicots on the transport of signalling compounds. For example, applied jasmonates accumulated extracellularly in tomato but intracellularly in barley (Bucking *et al.,* 2004). In contrast to dicots, PR1 genes may not be markers of induced resistance in wheat.

We used ERK specific inhibitor with SA to treat wheat leaves in order to examine if SA-induced resistance is downstream of ERK-type MAPK in wheat, and PR1.2 was chosen as the marker gene. Since exogenous SA did not have any effect on wheat PR1.2, we can't verify our hypothesis by using ERK specific inhibitor. Our results indicate that PR1.2 did not respond to ERK specific inhibitor, whereas PR1.2 transcript was decreased by general kinase inhibitor K252b, suggesting that PR1.2 may not be downstream of ERK-type MAPK pathway but rather downstream of other kinase pathways in wheat.

3.4 Material and methods

3.4.1 Plant materials and growth conditions

Frontana and Roblin seeds were originally obtained from Cereal Research Centre, Agriculture and Agri-Food Canada, Winnipeg, MB, Canada. Seeds were surface sterilized for 2 min in 70% ethanol and then soaked for 8 min in sterilization solution (25% Bleach v/v and 0.01% Triton X-100 v/v). Seeds were then rinsed 10 times with autoclaved water. After sterilization, seeds were sowed directly to autoclaved soil (BM1, Montreal, Quebec, Canada). They were then grown in 16 hours of light at 17°C and 8 hours of dark at 15°C in growth chambers (ENCONAIR Technologies Inc, Winnipeg, Manitoba). Each pot of wheat was fertilized with 7-9 ml granular 14:14:14 slow release fertilizer (NUTRICOTE Plant Product Co, LTD, Brampton, Ontario, Canada).

3.4.2 SA or FB1 treatment

To investigate the phosphorylation levels of ERK-type MAPKs in wheat leaves with SA and FB1 treatment, Frontana and Roblin leaves were collected and treated with SA and FB1 for indicated period of times.

Three to four leaves harvested from the 3-week-old plants were cut to ~2cm segments and incubated in 100μM SA (or 5μM FB1) solution under the same growth conditions after infiltration. Leaf segments were placed upward on the filter paper in Petri dishes so that they were just covered by the solution. Leaf segments treated in the same way with an equal volume of distilled water served as controls. Samples after 24 h, 48 h and 72 h incubation were collected. All of the leaf segments were collected in Falcon tubes and snap-frozen in liquid nitrogen. They were stored at -80°C and used for RNA or protein extraction within 3 months.

3.4.3 Protein isolation

Wheat protein was extracted from frozen leaves using TRIzol Reagent kit (Invitrogen, USA). Leaf tissue (100 mg) was homogenized in 1mL of TRIzol. The homogenized sample was incubated for 5 min at RT to permit the complete dissociation of nucleoprotein complexes. An aliquot of 200 µL of chloroform was then added to the sample, mixed vigorously by hand for 15 seconds, and incubated at RT for 2-3 min. The sample was centrifuged for 15 min at 12,000 x g at 4°C. Following centrifugation, the mixture separated into a lower red, phenol-chloroform phase, and an interphase, and a colorless upper aqueous phase. Protein remains exclusively in the interphase. The clear, aqueous top phase contains RNA, and the lower phenol-chloroform phase consists of DNA and other heavier products such as extracellular membranes and polysaccharides.

The aqueous phase was completely removed and saved for RNA extraction. DNA was precipitated with ethanol from the interphase and organic phase. An aliquot of 0.3mL of 100% ethanol was added, and the samples were mixed by inversion. Then the samples were stored at 15 to 30°C for 2-3 min followed by centrifugation at no more than 2,000 x g for 5 min at 2 to 8°C. Precipitated DNA was stored at -20°C and ready for use.

Proteins were precipitated from the phenol-ethanol supernatant obtained after precipitation of DNA with ethanol. 1.5 mL isopropanol was used for the initial homogenization. Samples were stored for 10 min at 15 to 30°C, and the protein precipitate was sediment at 12,000 × *g* for 10 min at 2 to 8°C. The supernatant was removed and the protein pellet was washed 3 times in 2 mL of solution containing 0.3 M guanidine hydrochloride in 95% ethanol. During each wash cycle, the protein pellet was stored in the wash solution for 20 min at 15 to 30°C and centrifuged at 7,500 × *g* for 5 min at 2 to 8°C. After the final wash, the protein pellet was vortexed in 2 mL of 100% ethanol. The protein pellet was stored in ethanol for 20 minutes at 15 to 30°C and centrifuge at 7,500 × *g* for 5 min at 2 to 8°C. The protein pellet was vacuum-dried for 5 to 10 min, and dissolved in 1% SDS by pipetting. Complete dissolution of the protein pellet requires incubating the sample at 50°C for 10 minutes. Then the insoluble material was sedimented by centrifugation at 10,000 x g for 10 min at 4°C. The supernatant was

saved and transferred to a fresh tube. The protein sample was stored at -20°C and ready for use in western blot.

3.4.4 Determination of protein concentration

Protein concentration in tissue extracts were determined using the Coomassie blue dye binding method with the Bradford reagent and bovine serum albumin (BSA) as the standard (Bradford, 1976). 200 mL of Bradford reagent was prepared by dissolving 20mg Coomassie Blue G250, 10 mL 95% ethanol, 20 mL 85% phosphoric acid and distilled water to dilute to 200 L. The Bradford reagent was filtered just before use. A 10 mg/mL solution of BSA (mix with 1%SDS) was diluted to make BSA standard curve as Table 3-1.

20 μL of each dilution and 1mL Bradford dye reagent were placed in 1.5 mL tube, and votexed. After 5 min, 1 mL of each dilution was transferred to a UV spectrometer cuvette. Absorbance at 595nm was measured for each dilution. Then a BSA standard curve was generated by plotting Abs595 against BSA concentration. In the same way, protein sample with Bradford dye reagent was placed in cuvette, and absorbance at 595nm was read. Concentration of protein sample was determined from the BSA standard curve.

Table 3-1: Dilution for bovine serum albumin (BSA) standard curve.

Final BSA concentration (mg/mL)	10mg/ml BSA volume (μL)	1%SDS (μL)
0.25	2.5	97.5
0.50	5.0	95.0
0.75	7.5	92.5
1.00	10.0	90.0
1.25	12.5	87.5

3.4.5 One-dimensional SDS-polyacrylamide gel electrophoresis

SDS-PAGE gel usually has two parts, the separating gel and the stacking gel. For separating gel, 2.5 mL of separating solution was added to 4.16 mL of dH_2O, and 3.3ml of 30% acrylamide/bisacrylamide stock solution. This solution was allowed to settle for fifteen minutes. While the solution was settling the plates were assembled. The glass plates were pressed together and then loaded into the gel casting apparatus. Once the plate was ready then 0.03 mL of 10% ammonium persulfate and 6.6 μL of TEMED were added to the settled solution. The final solution was then mixed by gently swirling the solution. Using a 1000 μL pipette the finished solution was added into the space between the glass plates until the solution was about 1.5 cm from the top of the plates. The gel was then overlaid with 10 μL of isobutyl alcohol and allowed to polymerize for one hour. While this was occurring the stacking gel was generated.

To create the stacking gel, 0.65 mL of 30% acrylamide/biscrylamide, 1.25 mL of stacking solution, and 3.05 mL of water were mixed together and allowed to settle. After the separating gel had solified, 25 μL of ammonium persulfate and 5 μL of TEMED were added to the separating solution and swirled gently to mix. The isobutyl alcohol was removed from the top of the separating gel and the stacking gel solution was added to the gel casting apparatus using a 1000 μL pipette. The comb was inserted and the gel was allowed to solidify for one hour. The gels were stored at 4°C until needed.

The concentration of protein samples were adjusted to 0.8 μg/μL with 2x SDS-PAGE sample buffer containing 100mM Tris-HCl (pH 6.8), 4% w/v lauryl sulphate (SDS), 20% v/v glycerol, 10% v/v β-mercaptoethanol and 0.2% w/v bromophenol blue, and the sample was then heated for 10 min at 90°C. Equal amounts of proteins from water control, SA treated and FB1 treated samples (8 μg) were then loaded into each well of 10% SDS polyacrylamide gels. Prestained SDS-PAGE standards (Bio-Rad, USA) were also loaded in one well to estimate the size and positions of sample proteins on the gel. Proteins were separated using the discontinuous buffer system of Laemmli (1970). Electrophoretic separation was generally carried out in 1X running buffer (3.03g Tris base, 14.4g glycine and 1g SDS per liter, pH~8.3) at 175V for ~50 min at RT using the

BioRad Mini-PROTEAN 3 System. After electrophoresis, proteins were transferred onto Immun-Blot polyvinylidene difluoride (PVDF) membranes (Bio-Rad, USA) by wet transfer with pre-chilled transfer buffer containing 25 mM Tris (pH 8.5), 192 mM glycine, and 20% v/v methanol at 4°C for 120 min at 70V.

3.4.6 Immunoblotting and protein visualization

To prevent non-specific binding, PVDF membranes were blocked with 5% non-fat milk in TBST (20M Tris, pH 7.5, 150Mm NaCl, 0.05% Tween-20) for 30 min at RT. The blots were washed with TBST (5 min x 3) and then incubated with 10 mL TBST buffer containing primary antibody against the protein of interest on a shaking platform at 4°C overnight. The primary antibody used was Phospho-p44/42 MAP Kinase (Erk ½) (Thr-202/Tyr-204) antibody at 1:1000 v:v (Cell Signaling Technology, USA). After overnight incubation with primary antibody, blots were washed with TBST (5 min x 3) and then incubated at RT for 1 h with 1:2000 v:v dilution of the secondary antibody (anti-rabbit IgG, HRP-linked) (Cell Signaling Technology, USA). The target protein on the PVDF membrane was detected using an enhanced chemiluminescence (ECL) system containing 1X LumiGLO Reagent and 1X Peroxide (Cell Signaling Technology, USA). The membrane was scanned using FluorChem Q imaging system (Alpha Innotech Cooperation, USA) and the resulting image was analyzed with the FluorChem Q Band Analysis tools. To confirm equal loading in each lane of the blot, total protein on the PVDF membrane was then visualized by staining for 10 min with Coomassie blue solution (0.25% w/v Coomassie Brilliant Blue R, 50% v/v methanol and 7.5% v/v acetic acid) followed by destaining for 30 min with destain solution (20% distilled water, 20% acetic acid, 60% methanol.

3.4.7 Quantification

Band intensities for the immunoreactive proteins of interest in water control, SA-treated and FB1-treated samples were quantified using FluorChem Q Band Analysis tools.

Intensities of Coomassie blue stained standard bands around 60kDa region were also quantified in each lane. Normalized band intensities were then determined as the ratio of immunoreactive band intensity versus Coomassie blot band intensity. Data were then plotted as histograms.

3.4.8 RT-PCR

Three-week-old wheat leaves were treated by distilled water, 100μM SA, 100μM SA with 250μM ERK docking domain inhibitor (3-(2-Aminoethyl)-5-((4-ethoxyphenyl) methylene)-2, 4-thiazolidinedione hydrochloride, Sigma, USA) and 100μM SA with 0.4μM general kinase inhibitor (K252b solution, Sigma, USA). Total RNA was extracted from wheat leaves (Frontana and Roblin cultivars) after treatment for 0h, 48h and 72 h. TRIzol Reagent kit (Invitrogen, USA) was used for RNA extraction according to manufacturer's protocol. cDNA was obtained according the protocol described previously in section 2.4.4. The primers used for RT-PCR were, 5'-CGTCTTCATCACCTGCAACTA-3' and 5'-CAAACATAAACACACGCACGTA-3' for PR1.2 (GenBank accession number AJ007349). A wheat actin gene (GenBank accession number AB181991) with the primer sequences 5'-GCCACACTGTTCCAATCTATGA-3' and 5'-TGATGGAATTGTATGTCGCTTC-3' was used as a positive control. RT-PCR was carried out under the following conditions: 94°C for 1 min; 1min at 94°C, 1min at 61°C, and 1min at 72°C for 25 cycles; and then 10 min at 72°C. Size for wheat PR1.2 gene PCR product is 144 bp. Size for the wheat actin gene PCR product is 369 bp.

Chapter IV

Programmed Cell Death in *Triticum aestivum*

4.1 Introduction

Programmed cell death (PCD) is recognized as an essential physiological and genetic process during plant development and in response to biotic and abiotic stresses (Beers and McDowell, 2001). Localized cell death occurs in both susceptible and immune plants during pathogen attack. In immune plants, a host resistance (R) protein recognizes a pathogen avirulence (Avr) protein leading to hypersensitive response (HR), which is a form of localized programmed cell death (Pozo *et al.*, 2004). Many host responses precede the HR, including proteolysis, changes in ion fluxes, production of reactive oxygen, and activation of MAPK cascades. In susceptible plant, much less is known about the molecular events leading to cell death. Studies suggest that host-controlled PCD plays a role in cell death occurred in different plant tissues (Greenberg and Yao, 2004). For biotrophic pathogens, early activation of host PCD would likely limit pathogen spread, whereas necrotrophic pathogens benefit from host cell death and kill the host by injecting toxins or activating host PCD (Pozo *et al.*, 2004).

The MAPK cascade is a major conserved signaling pathway used by host cells to transduce extracellular stimuli into intracellular responses in eukaryotes (MAPK Group, 2002; Nakagami *et al.*, 2005). Several studies have indicated that MAPKs are involved in regulation of cell death during plant-pathogen interactions (Pozo *et al.*, 2004; Rudd *et al.*, 2008; Yang *et al.*, 2001; Zhang and Liu, 2001). Mekk1 homozygous knockout plants show a severe dwarfism, accumulate high amounts of reactive oxygen species (ROS), and develop local lesions reminiscent of PCD (Ichimura *et al.*, 2006; Nakagami *et al.*, 2006). A tobacco MAPKK NtMEK2 is upstream of both WIPK and SIPK (Yang *et al.*, 2001). Expression of the active mutant of NtMEK2 induces HR- like cell death, defense gene expression, and ROS generation by activation of endogenous WIPK and SIPK (Yang *et al.*, 2001; Ren *et al.*, 2002). Moreover, del Pozo *et al.* (2004) identified a tomato MAPKKK, MAPKKKα, as a positive regulator of cell death associated with both plant

immunity and disease. Recently, Takahashi *et al.* (2007) identified a MAPKK gene (NbMKK1) as a potent inducer of the HR-like cell death by performing a high-throughput overexpression screen of *Nicotiana benthamiana* cDNAs. They demonstrated that MAPK cascades involving NbMKK1 control non-host resistance (Takahashi *et al.*, 2007).

Programmed cell death is often associated with concurrent accumulation of ROS and salicylic acid (Pitzschke *et al.*, 2009). ROS are the partially reduced or activated derivatives of oxygen. They are the highly reactive by-products of aerobic metabolism (Pitzschke and Hirt, 2009). MAPK signaling pathways are not only induced by ROS but can also regulate ROS production. Phenotypic and molecular analysis revealed that the MAPK kinases MKK1 and MKK2 are part of a cascade, regulating ROS and SA accumulation (Pitzschke *et al.*, 2009). Moreover, Liu *et al.* (2007) indicated that the MAPK cascade actively blocks carbon fixation, leading to an excess of excitation energy in illuminated plants and consequently accumulation of ROS. The sustained generation of ROS cannot be compensated for by the action of antioxidant synthesis enzymes. Therefore, after depletion of the antioxidant pool, ROS leads to cell death. These studies suggest that ROS plays an intermediary role between MAPK cascade and PCD. As a response to pathogen attack, ROS can trigger the deposition of lignin and callose, which serve to reinforce cell walls surrounding infection sites in order to halt pathogen invasion (Pontier *et al.*, 1998). So, the callose deposition is a signature feature of PCD in plants.

Specific DNA fragmentation into oligonucleosomal units or DNA laddering occurs during PCD in both animal and plant cells (Danon *et al.*, 2000). DNA laddering phenomenon is the product of chromatin digestion catalyzed by nucleases but without protease involvement (i.e. no histone digestion). Necrosis and a DNA smear on agarose gels are normally caused by concurrent nuclease and protease activity (Wyllie *et al.*, 1980). DNA laddering phenomenon has been studied in different plant developmental processes and under environmental stresses such as cold (Koukalovà *et al.*, 1997), UV radiation (Danon and Gallois, 1998), and heat (Balk *et al.*, 1999, Swidzinski *et al.*, 2002). Harsh environmental stresses often cause necrosis that is an accidental cell death, accompanied with DNA smear, rupture of nuclear, organelle and plasma membranes

(Danon *et al.,* 2000). Therefore, DNA ladders are currently used to distinguish apoptosis from necrosis at the molecular level (Danon *et al.,* 2000).

As previous studies have shown, stress, SA, ROS, and MAPK cascades are strongly interconnected (Nakagami *et al.*, 2005; Pitzschke and Hirt, 2006). Programmed cell death is believed to be mediated in part by SA (Dat *et al.*, 2007). The evidence for a modulating role of SA during PCD is that addition of SA to either soybean suspension cultures or *Arabidopsis lsd1* mutants accelerated cell death (Dietrich *et al.,* 1994; Shirasu *et al.,* 1997). The characterization of two other *Arabidopsis* mutants *acd* and *lsd* has also supported the crucial role of SA in cell death processes, because both mutants have constitutively high SA levels (Rate *et al.,* 1999; Brodersen *et al.,* 2002). Moreover, exogenous application of SA in *acd11/eds5-1 Arabidopsis* mutants also accelerated PCD (Brodersen *et al.,* 2005).

Fumonisin B1 induces apoptosis-like PCD in both plants and animals (Asai *et al.*, 2000). In *Arabidopsis*, FB1 treatment initiates nuclear DNA fragmentation preceding the loss of membrane integrity, which resembles apoptosis typically associated with PCD in animal cells. FB1 induced cell death is dependent on active transcription and translation, as well as reversible protein phosphorylation (Asai *et al.*, 2000). Asai *et al.* (2000) have proposed a model for cell death signaling in *Arabidopsis* protoplasts induced by FB1. They demonstrated that FB1-elicited PCD requires SA, jasmonic acid (JA) and ethylene (ET)-dependent signaling pathways as well as one or more unknown factors activated by FB1. Our recent study has indicated that *Arabidopsis* ethylene receptors have different roles in FB1-induced cell death (Plett *et al.*, 2009).

As shown in the previous chapter, SA and FB1 enhanced the phosphorylation of ERK-type MAPKs in Frontana, a FHB resistant wheat cultivar. It has been suggested that there is a correlation between MAPK activity and cell death during plant-pathogen interactions (Pozo *et al.*, 2004; Yang *et al.*, 2001; Zhang and Liu, 2001). In this chapter, we have shown that SA and FB1 induce programmed cell death in both resistant and susceptible wheat cultivars (Frontana and Roblin, respectively) by trypan blue detectin and DNA laddering analysis. To determine whether the cell death detected by trypan blue

assay is associated with callose deposition, we determined the presence of callose with aniline blue.

4.2 Results

4.2.1 SA and FB1 induce programmed cell death in Frontana and Roblin cultivars

Cell death occurred on leaves detached from three-week-old Frontana and Roblin when they were treated with water, 5 μM FB1 and 100 μM SA and incubated in long day conditions for up to 72 h. Trypan blue is commonly used to selectively stain dead tissues or cells blue, and under white light the dead cells appeared to be much darker compared to living cells. These blue dead cells scattered on leaves as clusters without defined margins and the cell death did not seem to occur in the whole leaf. Microscopic images were taken by focusing on one of several dead cell groups. Leaves were collected from leaves treated with FB1, SA or water for 0, 24, 48, and 72h. Water treatment was used as a control for all plants (Figure 4-1-d). Cell death happened on leaf veins upon all of the treatments (Figure 4-1-a, b and c), but cell death outside of the leaf vein only appeared when leaves were treated with FB1 and SA (Figure 4-1-e and f), and no dead cells were detected in the water control (Figure 4-1-d). Upon FB1 and SA treatment, cell death was first observed at 24 h in both Frontana and Roblin, and more death cells were detected when leaves were incubated longer (Figure 4-1-e and f).

To determine whether the cell death detected by trypan blue assay is associated with callose deposition, we determined the presence of callose with aniline blue in Frontana and Roblin leaves 0h, 24h, 48h, and 72h after SA or FB1 treatment. The accumulation of callose deposition around the site of infection is usually part of the complex cell wall-strengthening process that halts pathogen invasion (Stanghellini and Aragati, 1996). The stain of aniline blue is used to detect callose deposition and produces UV-stimulated fluorescence (Eschrich and Currier, 1964). The fluorescence produced by callose appears as a bright blue fluorescent ring and is distinct from the white autofluorescence under the epifluorescence microscope (Figure 4-1-g, h, i). In water control, cell death was induced

at 72 h, and the area of dead cells is larger on Roblin leaves than on Frontana leaves (Figure 4-1-g). The earliest cell death induced in SA and FB1 were at 24 h in both Frontana and Roblin, and more cell death appeared at 72 h (Figure 4-1-h and i).

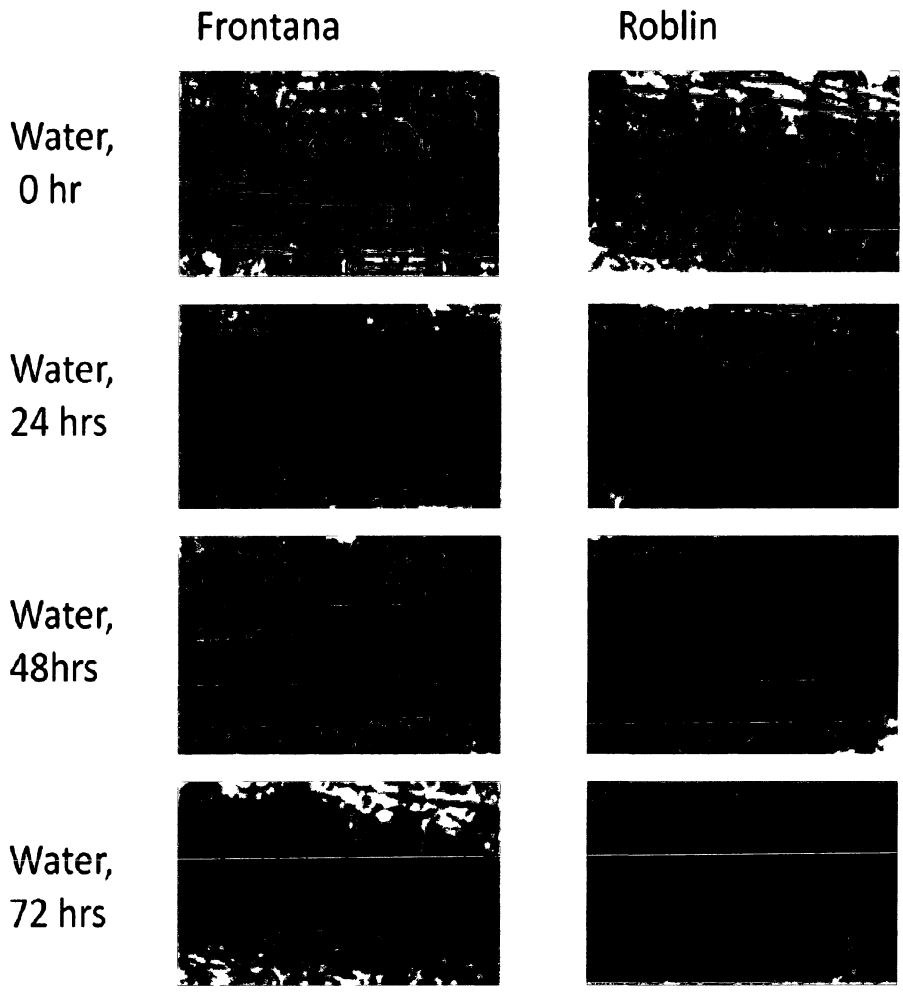
Overall, upon fungal toxin fumonasin B1 (FB1) treatment, cell death occurred earlier in Frontana leaves than in Roblin leaves as detected by trypan blue and aniline blue, and there were more cell death in Roblin leaves than in Frontana leaves at 48h and 72h as indicated by aniline blue assay. Macroscopic data are shown in Figure 4-2.

Figure 4-1: Microscopic images of Frontana and Roblin leaves.

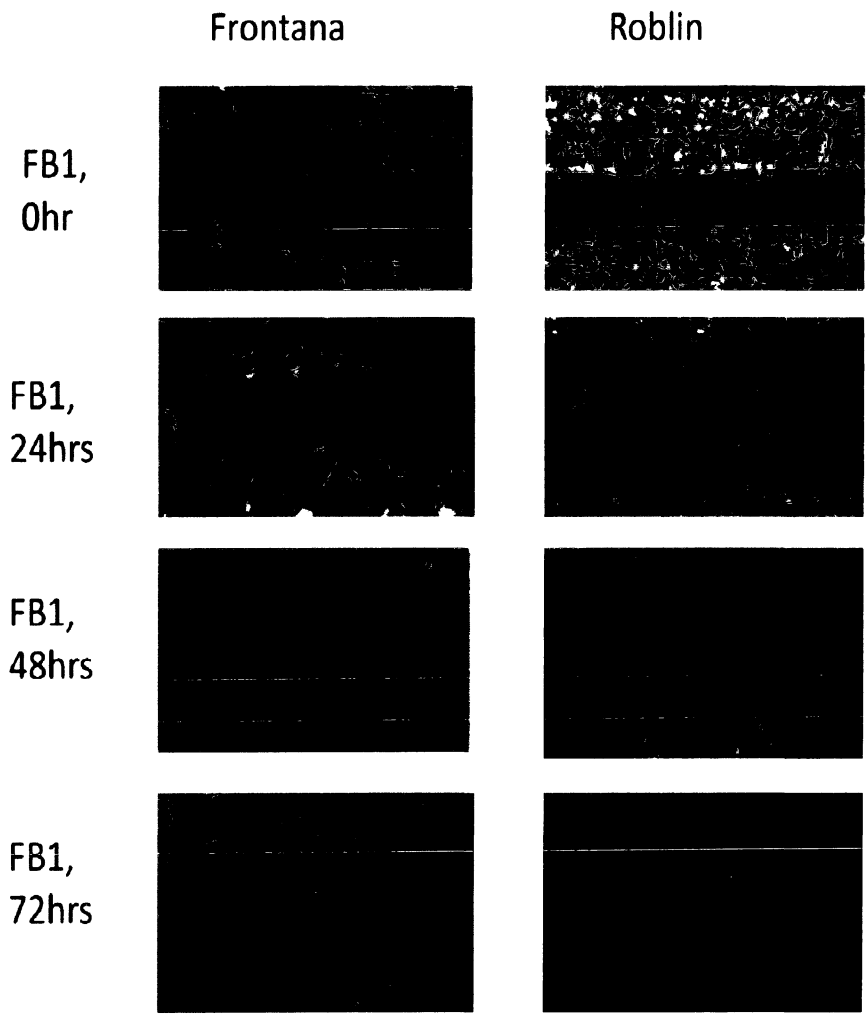
All plants were grown in a growth chamber under long day conditions (16h light/8h dark). Leaves detached from three-week-old plants were treated with water, 5μM FB1 and 100μM SA. Half of each type of leaves was then stained with trypan blue. A microscopic image was taken with white light by Axioplan 2 microscope. The other half portion of leaves was stained with aniline blue. A microscopic image was taken using a microscope equipped with a UV light source and DAPI filter (scan range: 320-520 nm). Arrows indicate the sites of stain or fluorenscent signals. Only one experiment was carried out.

a) Cell death on Frontana and Roblin leaf veins under the control (water) treatment by trypan blue assay.
b) Cell death on Frontana and Roblin leaf veins under 5μM FB1 treatment by trypan blue assay.
c) Cell death on Frontana and Roblin leaf veins under 100μM SA treatment by trypan blue assay.
d) Cell death outside of Frontana and Roblin leaf veins under the control (water) treatment by trypan blue assay.
e) Cell death outside of Frontana and Roblin leaf veins under 5μM FB1 treatment by trypan blue assay.
f) Cell death outside of Frontana and Roblin leaf veins under 100μM SA treatment by trypan blue assay.
g) Cell death on Frontana and Roblin leaves under the control (water) treatment by aniline blue assay.
h) Cell death on Frontana and Roblin leaves under 5μM FB1 treatment by aniline blue assay.
i) Cell death on Frontana and Roblin leaves under 100μM SA treatment by aniline blue assay.

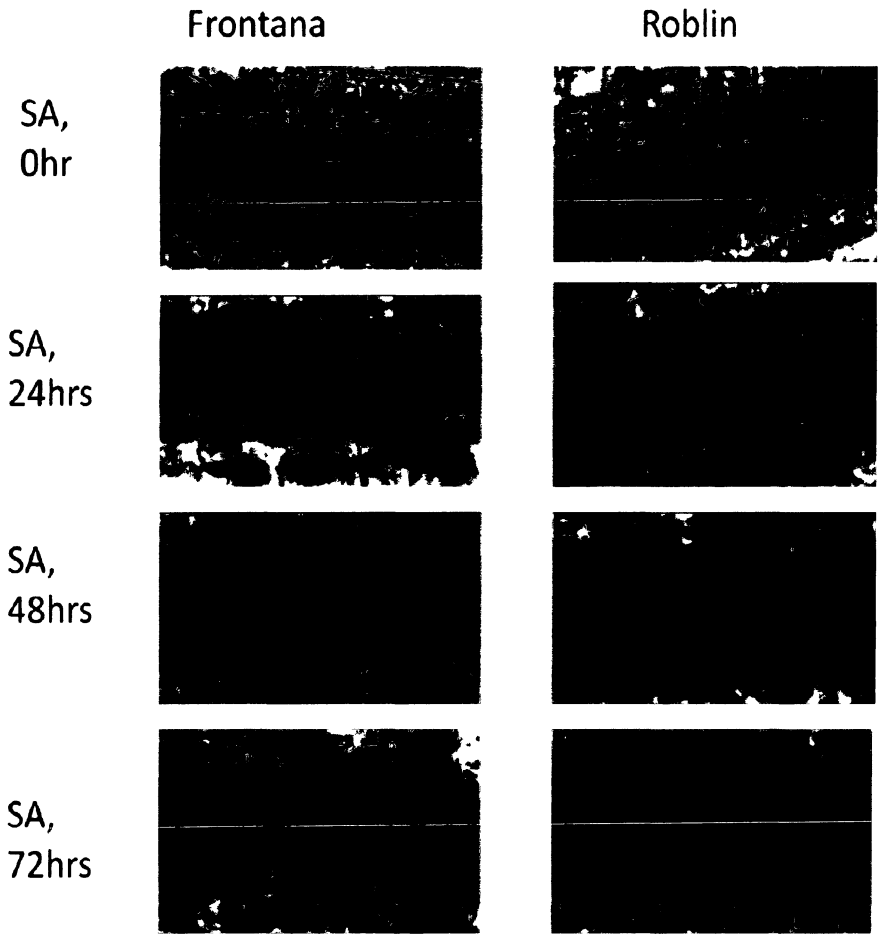
a) Water, on leaf veins



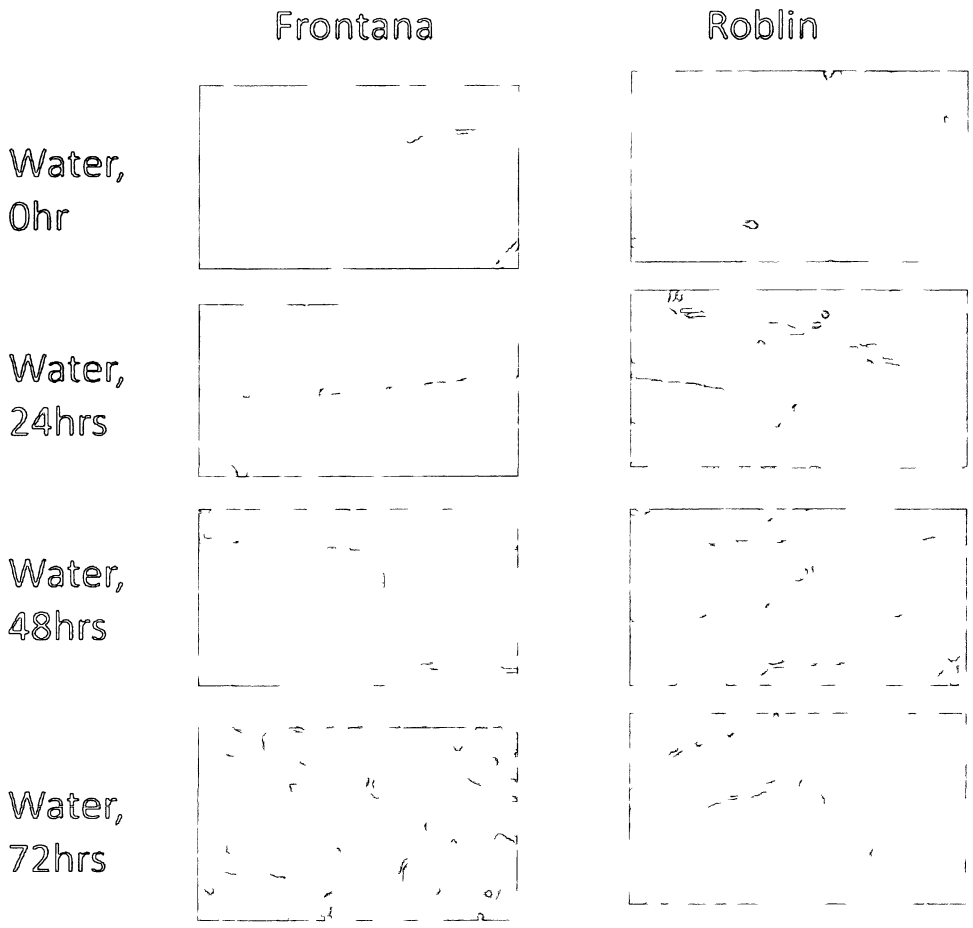
b) 5μM FB1, on leaf veins



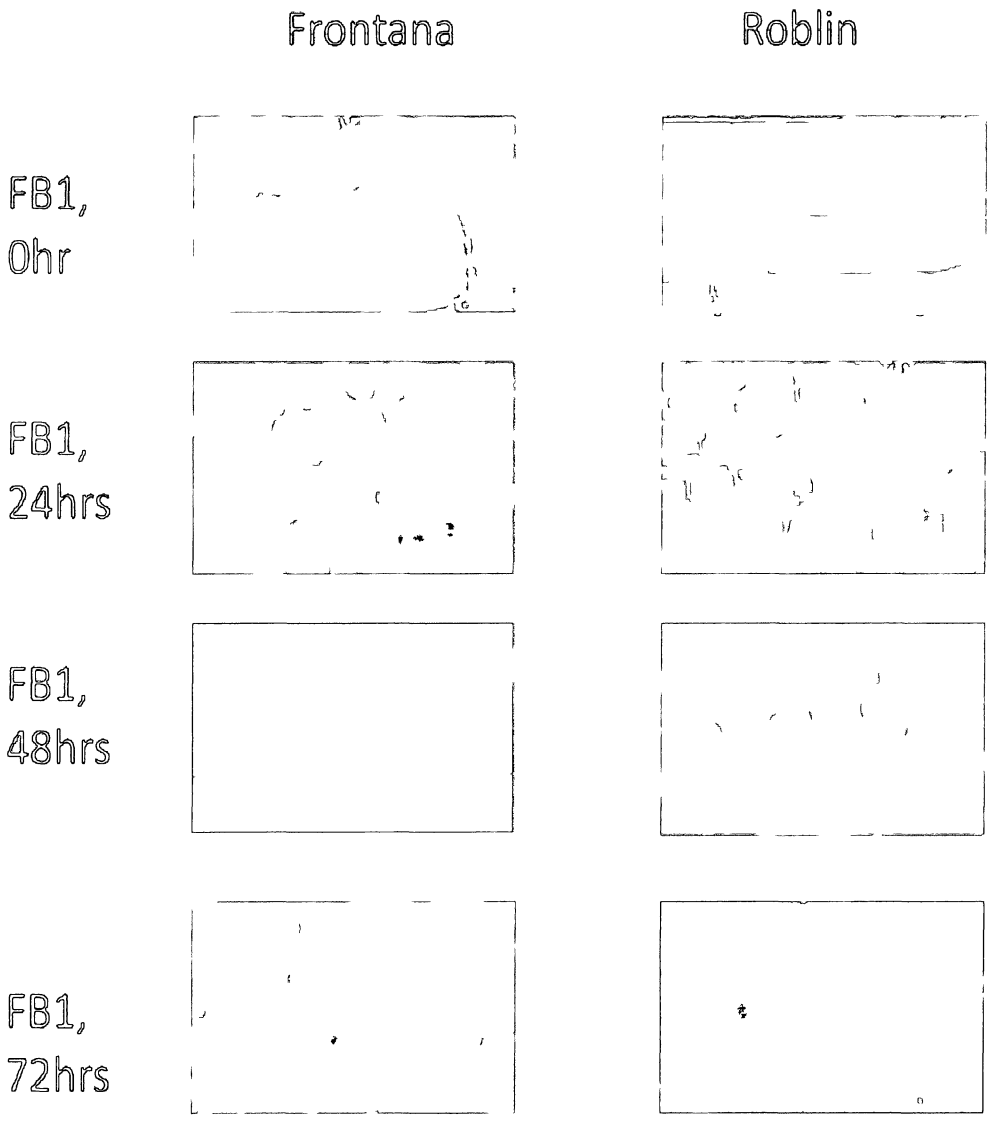
c) 100 μM SA, on leaf veins



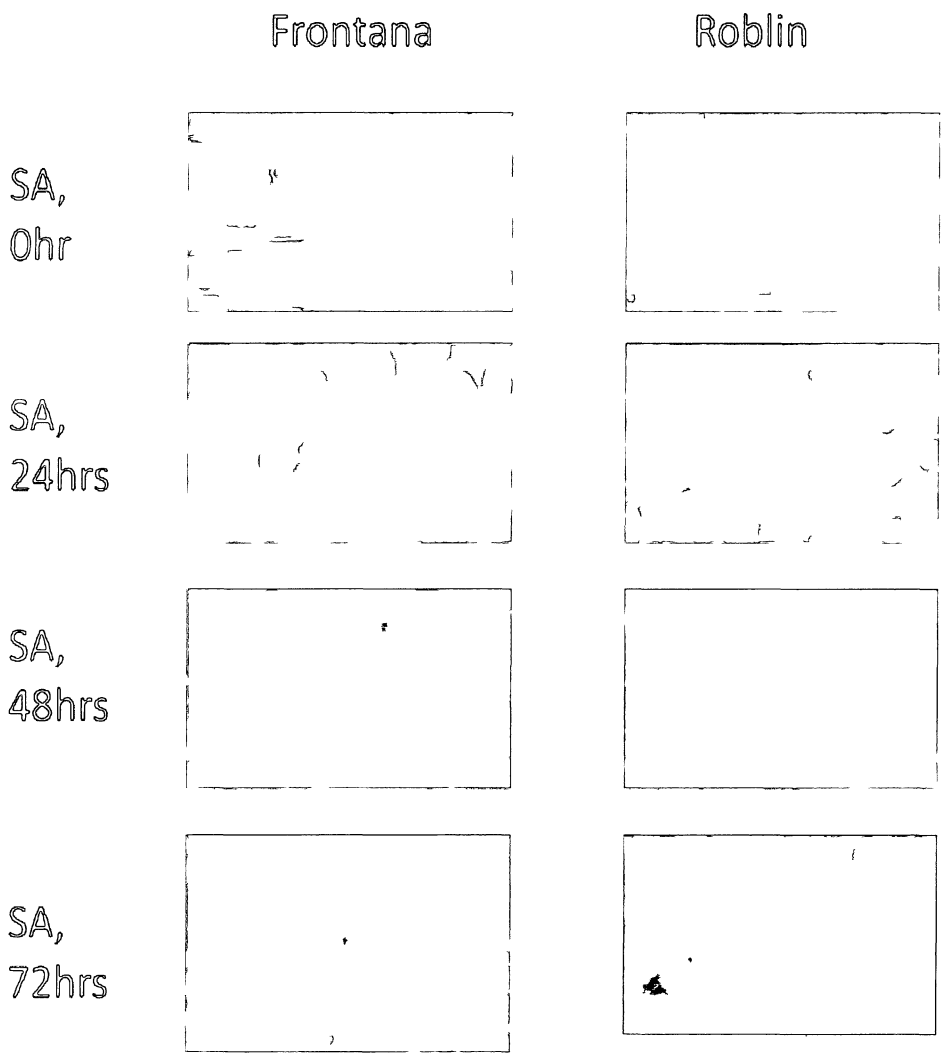
d) Water, outside of leaf veins



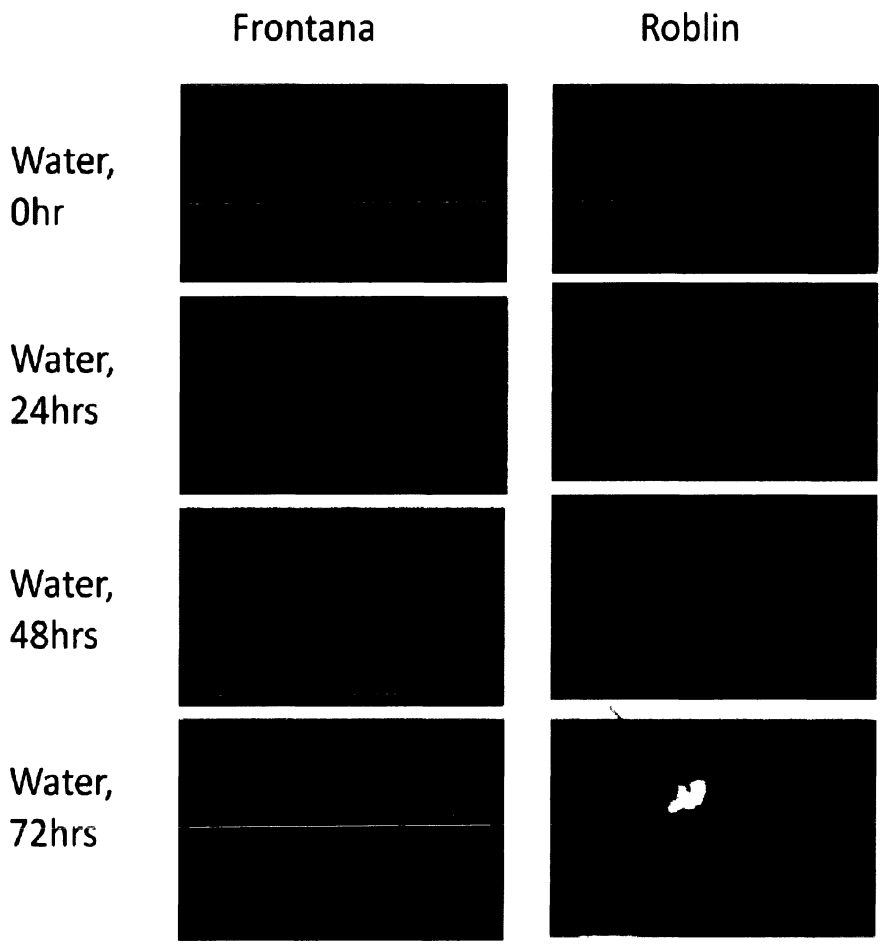
e) 5μM FB1, outside of leaf veins



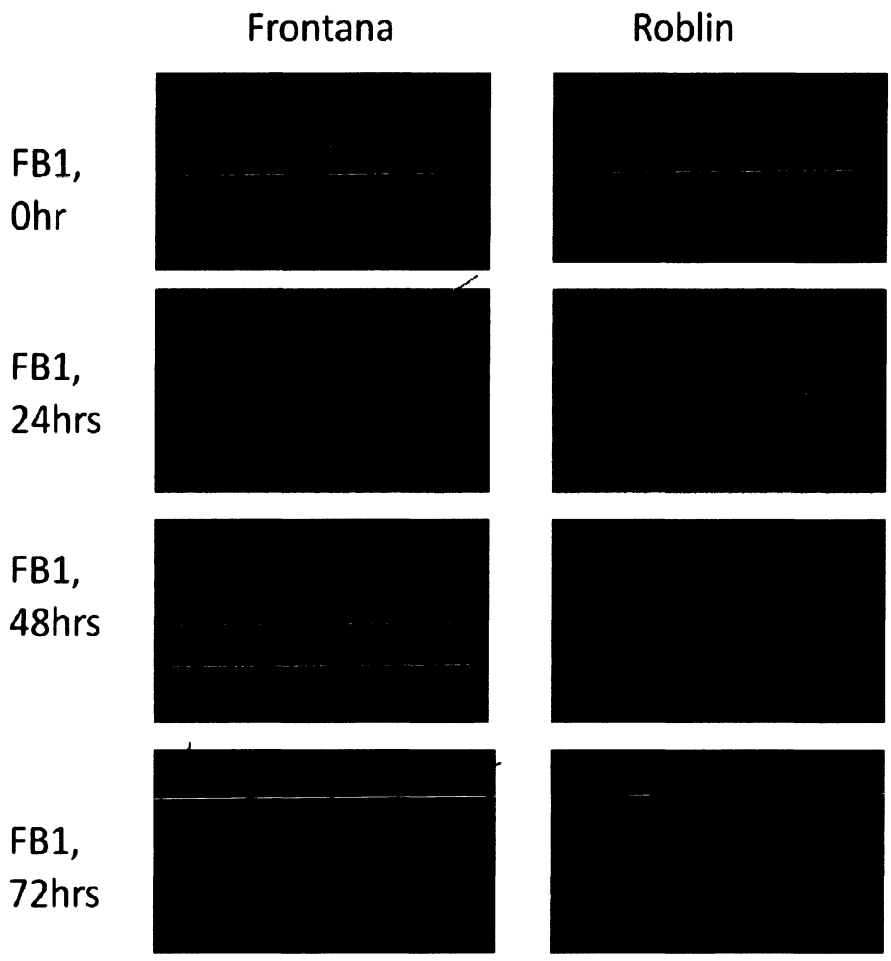
f) 100µM SA, outside of leaf veins



g) Water, aniline blue



h) 5μM FB1, aniline blue



i) 100μM SA, aniline blue

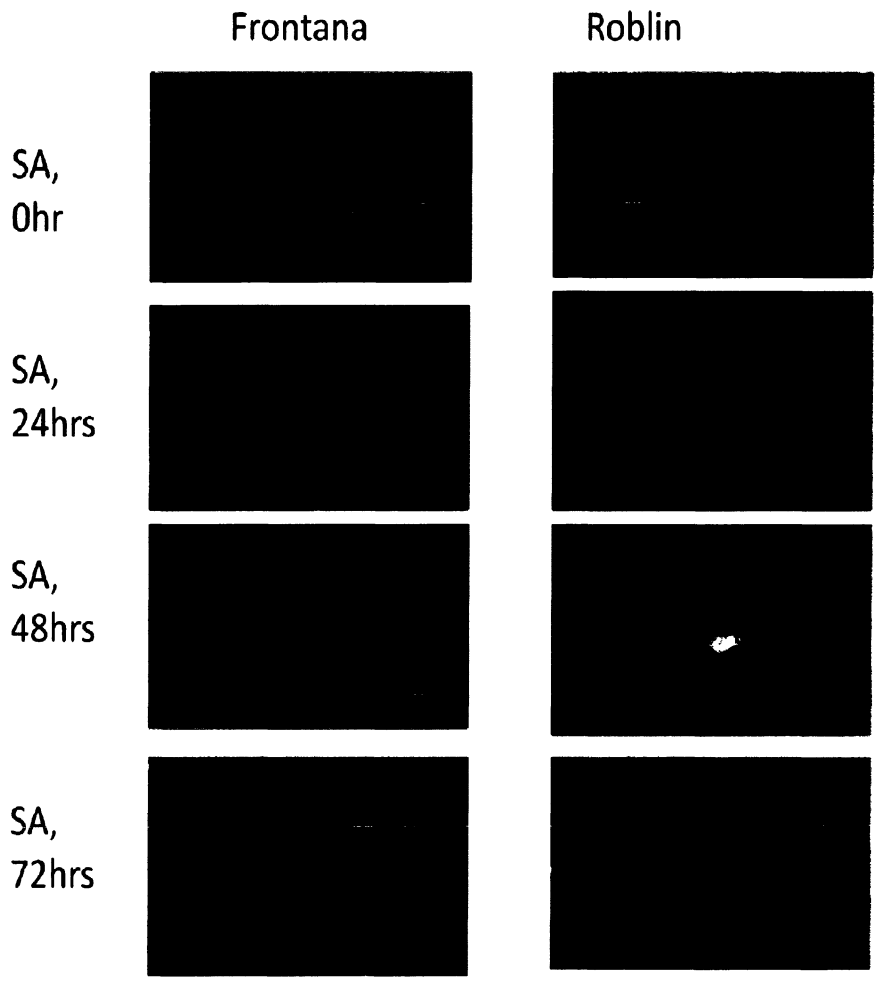
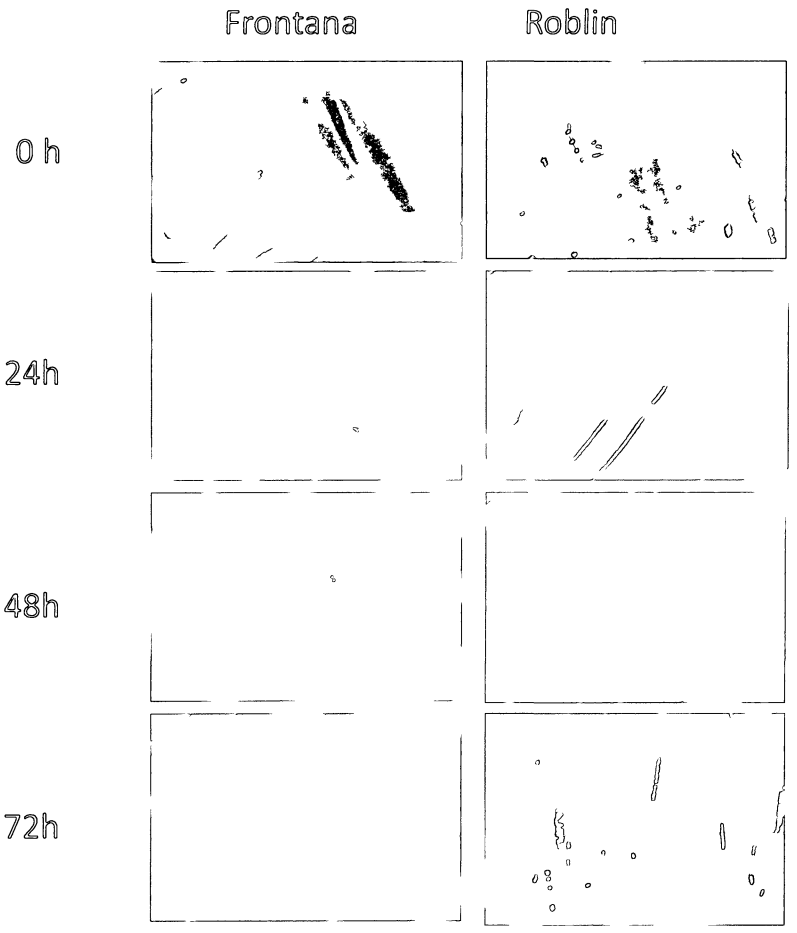


Figure 4-2: Macroscopic images of Frontana and Roblin leaves.

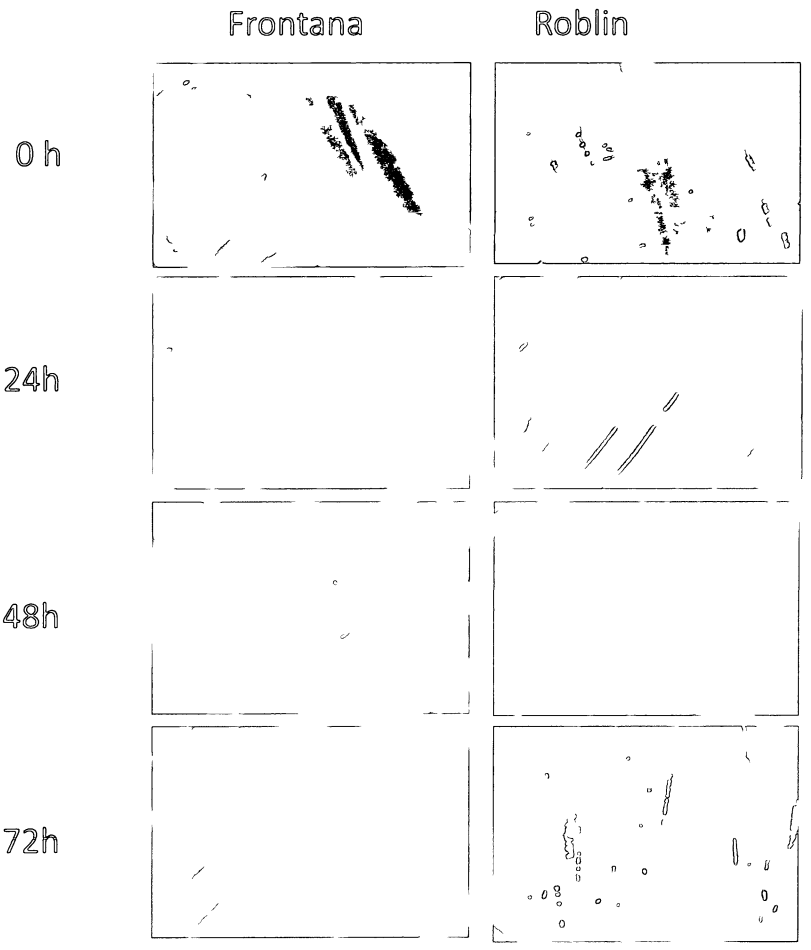
All plants were grown in a growth chamber under long day conditions (16h light/8h dark). Leaves detached from three-week-old plants were treated with 5µM FB1 and 100µM SA. A macroscopic image was taken with a digital camera. Only one experiment was carried out.

a) Frontana and Roblin leaves under 5µM FB1 treatment.
b) Frontana and Roblin leaves under 100µM SA treatment.

a) 5µM FB1



b) 100µM SA



4.2.2 SA and FB1 induce DNA smearing in Frontana and Roblin cultivars

Most of the current knowledge about PCD in plants has come from studies with cell culture systems (Ning *et al.*, 2002). DNA ladder phenomenon is one of the most common characteristics of PCD in animals and plants. The DNA ladder indicates that DNA fragmentation occurs while cell structures, such as vacuoles or lysosomes, are preserved at least during part of the cell death process (Danon *et al.*, 2000).

We monitored the integrity of DNA by electrophoresis in a 2% agarose gel. When fresh Frontana leaves were treated with FB1 for different periods of time, DNA isolated from untreated leaves and DNA from treated leaves both showed DNA smearing (Figure 4-3-a, *lane* 1-4). The strongest smearing was observed at 24 hours and 48 hours after treatment (Figure 4-3-a, *lane* 2, 3). SA treatment for 24 hours to 72 hours caused DNA smearing, and DNA smearing was more significant after 24-hour treatment than after 48-hour or 72-hour treatment (Figure 4-3-a, *lane* 5-7).

When fresh Roblin leaves were treated with FB1 and SA for different periods of time, a significant DNA smearing was observed (Figure 4-3-b). DNA isolated from untreated leaves migrated as an unresolved high molecular mass band of more than 12 kbp in length (Figure 4-3-b, *lane* 1). In contrast, DNA from leaves treated with FB1 for 24-72 hours (Figure 4-3-b, *lane* 2-4) showed DNA smearing. DNA smearing apparently increased with the time of FB1 treatment. After 72 hours, there was almost no intact DNA left (Figure 4-3-b, *lane* 4). When Roblin leaves were treated with SA, there was a significant DNA breakdown but giving no ladders, and DNA smearing increased with the time of SA treatment (Figure 4-3-b, *lane* 5-7).

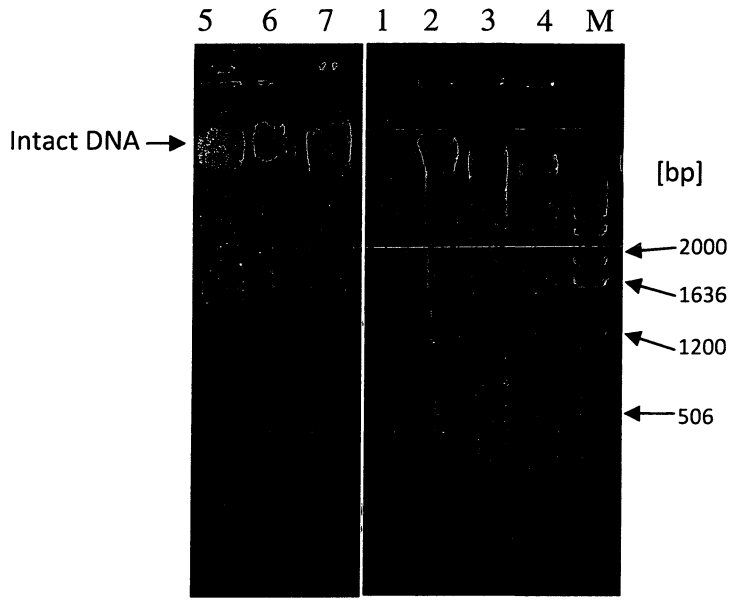
Figure 4-3: Wheat leaf DNA smearing after SA and FB1 treatments

Ten µg DNA per lane was loaded onto a 2% agarose gel followed by electrophoresis.

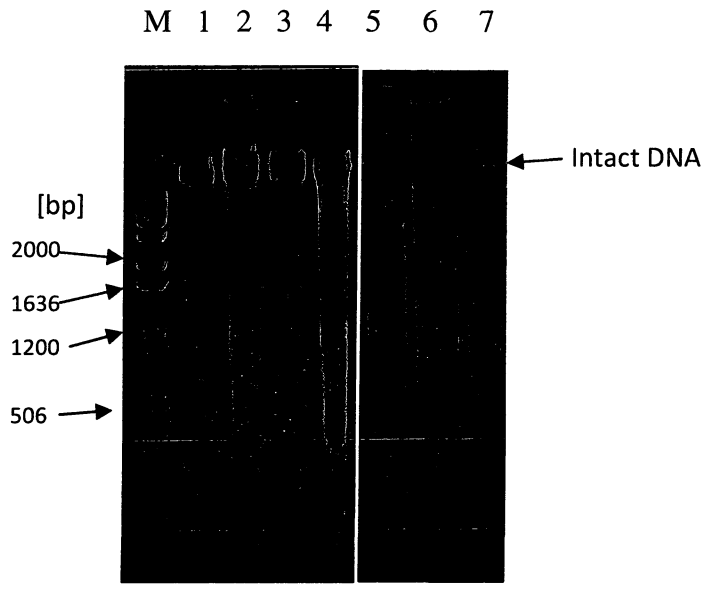
a) Frontana leaves DNA laddering and smearing after SA and FB1 treatments. *Lane* 1- untreated Frontana leaves, *lanes* 2 – 4 - Frontana leaves after FB1 treatment for 24, 48 and 72 hours, respectively, lanes 5 -7 – Frontana leaves after SA treatment for 24, 48 and 72 hours, respectively. M – DNA markers.

b) Roblin leaves DNA laddering and smearing after SA and FB1 treatments. *Lane* 1- untreated Roblin leaves, *lanes* 2 – 4 - Roblin leaves after FB1 treatment for 24, 48 and 72 hours, respectively, lanes 5 -7 – Roblin leaves after SA treatment for 24, 48 and 72 hours, respectively. M – DNA markers.

a)


5 6 7 1 2 3 4 M
Intact DNA
[bp]
2000
1636
1200
506

b)


M 1 2 3 4 5 6 7
[bp]
2000
1636
1200
506
Intact DNA

4.3 Discussion

In this chapter, we have analysed the SA and FB1 induced programmed cell death in wheat FHB resistant and FHB susceptible cultivars, Frontana and Roblin. In both of these cultivars, programmed cell death happened when wheat leaves were treated with SA and FB1.

4.3.1 Programmed cell death in wheat under SA and FB1 treatments

Plants mount numerous defense responses to survive pathogen attack. One of these is programmed cell death, which is characterized by the rapid death of plant cells at the site of pathogen infection (Lam, 2004). From the literature review, it is suggested that the plant hormone salicylic acid (SA) is essential for many plant defense responses, but under other circumstances it appeared that SA is not required. It is also believed that programmed cell death is mediated in part by SA (Dat *et al.*, 2007). Cell death occurs in both susceptible and resistant plants during pathogen attack (Pozo *et al.*, 2004). Our results showed that cell death occurred on both Frontana and Roblin leaves under SA treatment, suggesting that SA is involved in defense response in the form of cell death in both immune and susceptible wheat cultivars.

Fumonisin B1, a PCD-eliciting fungal toxin, is a sphinganine analogue that has been shown to trigger dosage-dependent cell death in *Arabidopsis* that shares many features with avirulent pathogen-induced cell death (Stone *et al.,* 2000; Asai *et al.,* 2000). We demonstrated that FB1 induces cell death in FHB susceptible and FHB resistant wheat cultivars. The results also showed that cell death occurred earlier in resistant wheat leaves than in susceptible leaves, suggesting that the particular host-pathogen interaction determines the rapidity of activation of defense responses. This can be explained by the fact that certain amounts of disease symptoms in susceptible plants appear over the course of days, whereas defense responses are induced within hours in 'gene-for-gene' immunity (Pozo *et al.,* 2004). Timely activation of cell death is necessary in some host-pathogen interactions for pathogen containment, whereas in other situations cell death is either not essential or not sufficient for disease resistance (Lam *et al.,* 2001). Our observations have shown more cell death occurred in Roblin leaves than in Frontana

leaves after 2 to 3 days treatment with FB1 as indicated in aniline blue assay, so it is possible that early activation of host PCD in Frontana would likely limit pathogen proliferation.

Upon SA and FB1 treatment, the levels of cell death detected by aniline blue assay and by trypan blue assay correlate well, suggesting that the cell death indicated in trypan blue assay is associated with callose deposition, which is triggered by ROS. Therefore, we demonstrated that programmed cell death in wheat is associated with concurrent accumulation of ROS.

4.3.2 ERK-type MAPK as a potential inducer of programmed cell death in wheat

Several studies have indicated MAPKs are involved in regulation of cell death during plant-pathogen interactions (Pozo *et al.*, 2004; Yang *et al.*, 2001; Zhang and Liu, 2001). In Chapter III, we showed that ERK-type MAPKs were activated in resistant wheat cultivar by SA and FB1 after 48-hour incubation (Figure 3-1), which was detected coincidently with the appearance of PCD on resistant wheat leaves (Figure 4-1) in this chapter. WCK1 (GenBank accession no AY079318) encodes an extracellular signal-regulated kinase (ERK)-like MAPK and shown to be transcriptionally activated in a fungal elicitor-treated cell culture (Takezawa, 1999). It would be interesting to see if ERK-type MAPKs play a regulatory role in SA- or FB1-induced cell death in wheat by using RNAi silencing approach (Smith *et al.*, 2000). If ERK-type MAPKs silencing abrogates the programmed cell death caused by SA or FB1, it would suggest that wheat ERK-type MAPKs are involved in cell death regulation.

4.3.3 DNA smearing in wheat under SA and FB1 treatments

Plants use the PCD process as one of many mechanisms that are required for the normal developmental elaboration of the plant life cycle (Gunawardena *et al.*, 2004). Developmental uses of PCD include (1) differentiation of specialized cell types such as

tracheary elements, (2) deletion of tissues with ephemeral functions, such as the embryonic suspensor, and (3) organ or shoot morphogenesis, such as the formation of functionally unisexual flowers from bisexual floral primordia (Greenberg, 1996; Jones and Dangl, 1996; Beers, 1997; Pennell and Lamb, 1997; Jones, 2000, 2001; Kuriyama and Fukuda, 2002). From our results, DNA smearing happened in untreated Frontana leaves, but not in untreated Roblin leaves. Also, there was development difference between Frontana and Roblin. For DNA laddering experiment, leaf samples were collected from three-week old wheat. At three weeks, it is possible that the vein system is more developed in Roblin than in Frontana and developmental PCD still occurs in Frontata leaves and DNA smearing could be detected. Further work on leaf development may verify this explanation.

SA and FB1 treatments triggered DNA smearing instead of laddering in wheat. DNA smear is observed in necrosis, which is the result of severe detrimental changes in the environment of affected cells and is not an active gene-dependent form of cell death (Danon *et al.,* 2000). Therefore, wheat leaves undergo necrosis instead of apoptosis with SA and FB1 treatment. Normally, concurrent nuclease and protease activity causes necrosis and a DNA smear on agarose gels (Wyllie *et al.,* 1980). It is possible that at some stage(s) nucleases and proteases are involved in SA and FB1 induced necrosis in wheat leaves.

Also, our results showed that intense DNA smearing was observed earlier in Frontana (a resistant cultivar) than in Roblin (a susceptible cultivar), but stronger smearing phenomenon appeared in Roblin leaves than in Frontana leaves after extended treatment. This is consistent with our microscopic results discussed in section 4.3.1. This provides another evidence that defense response is induced faster in resistant wheat cultivar than in susceptible cultivar. It may also indicate that early activation of PCD in wheat would likely limit disease proliferation, since DNA smearing became weaker after 48 hours of treatment in Frontana.

4.4 Materials and Methods

4.4.1 Plant materials and growth conditions

As described in section 3.4.1.

4.4.2 SA or FB1 treatment

To investigate PCD under SA and FB1 treatment, Frontana and Roblin leaves were collected and treated with SA and FB1 for different periods of time.

Three to four leaves harvested from the 3-week-old plants were cut to ~2cm segments and incubated in 100μM SA solution or 5μM FB1 at same growth condition after infiltration. Leaf segments were stretched upward on the filter paper in Petri dish so that they were just covered by the solution. Control leaf segments were treated in the same way with an equal volume of distilled water. Samples after 24 h, 48 h and 72 h incubation were collected. The leaf segments without any treatment were taken as the sample at 0h for both SA and water treatment.

All of the leaf segments were collected in Falcon tubes, and directly used for staining and DNA extraction.

4.4.3 Trypan blue and aniline blue staining

Cells of wheat leaves undergoing cell death were photographed using an Axioplan 2 microscope (Carl Zeiss, Germany). Methods described by Tang *et al.* (1999) and Stone *et al.* (2000) were used with slight modifications. Falcon tubes containing the leaves were boiled at 95°C for 4 min and kept at room temperature for 20 min instead of 30 min for the detection of autofluorescent materials.

Wheat leaves were immersed in 10 mL of ethanol-lactophenol (2 volumes of ethanol and 1 volume of phenol-glycerol-lactic acid-water (1:1:1:1)) that contained 0.05% trypan

blue. The leaves were placed in 15 ml Falcon tubes and covered with ethanol-lactophenol-trypan blue. The samples were incubated at 95°C for 4 min and then kept at room temperature for 20 min. The staining solution was removed and 1.5 mL chloral hydrate destaining solution (2.5 g/mL of nano pure water) was added to each tube. The leaves were cleared for 2 days by replacing the destaining solution twice. After destaining, leaves were suspended in 50% glycerol and examined under Axioplan 2 microscope (Carl Zeiss, Germany) with white light.

Wheat leaves were immersed and vacuum-infiltrated in 10 mL of ethanol-lactophenol (2:1 v : v) and then incubated at 60°C for 30 min. Leaves were rinsed in 50% ethanol and stained overnight with aniline blue (0.01% aniline blue powder in 150 mM K_2HPO_4, pH 9.5). Leaves were equilibrated in 50% glycerol and aniline blue staining was visualized using a UV epifluorescence (Axioplan 2 microscope, Carl Zeiss) with a DAPI filter.

4.4.4 Isolation of nuclear DNA and DNA laddering analysis

Total DNA was isolated using a modified method of Fan *et al.* 1998. Briefly, wheat leaves treated or untreated were ground to a fine powder in liquid nitrogen and added to the extraction buffer (200 mM Tris, pH 7.5, 25 mM EDTA, and 0.5% sodium dodecylsulphate). The supernatant was extracted with phenol and chloroform before precipitation with isopropanol. The DNA solution was incubated at 37°C for 1h in the presence of RNase and 10 µg DNA was separated by electrophoresis in a 2% agarose gel. The gel was then stained with ethidium bromide for visualization of DNA.

Chapter V

Overall Discussion

Plants respond to environmental stresses and pathogen attacks through the activation and coordination of various pathways. This activation and coordination require the phosphorylation of proteins, to date, many kinases, phosphatases and other molecules involved in phosphorylation pathways have been characterized (Xing *et al.*, 2002; Thurston *et al.*, 2005). Mitogen activated protein kinase (MAPK) pathway was one of the key phosphorylation pathways. In this pathway, MAPKKK activates MAPKK, which then activates MAPK. The active MAPK may allow the activation of other protein kinases, catalyze the phosphorylation of cytoskeleton components or activate transcription factors once translocated into the nucleus (Hirt, 1997). This pathway plays a role in responsiveness to a variety of stress such as pathogen attack, wounding and other abiotic stresses, as well as plant hormones such as ABA, auxins and ethylene (Hirt, 1997, Romeis, 2001; Xing *et al.*, 2002).

5.1 FLR and FRLS are MAPK pathway components

FLR and FLRS genes were discovered when we found they responded to wheat leaf rust challenge in preliminary wheat expressed sequence tag (EST) array analysis (He and Xing. unpublished). We have characterized the wheat FLR gene (AY173961), whose 1923bp cDNA encodes a novel 641-amino acid protein containing protein kinase catalytic domain, serine/threonine-protein kinase-like domain and the signature motif 'GTPEFMAPE' for one of the MAPKKK classes (ZIK1 class) (Cvetkovska *et al.*, 2005). The FLR protein is similar to *Homo sapiens* mitogen-activated protein kinase kinase kinase (PRKWNK2 gene) (AJ242724). Therefore, it is proposed that FLR is a MAPKKK gene in wheat. Also, we have analyzed the wheat FLRS gene (AY173962), whose 1212 cDNA encodes a novel 404-amino acid protein containing protein kinase domain and Thr-Glu-Tyr (T-X-Y) signature motif for MAPK in subdomain VIII. FLRS protein has

high similarity with MAP kinase 6 from *Oryza sativa* (japonica cultivar). It is well known that MAPKs are activated by phosphorylation on the Thr and Tyr residues in the activation loop having the consensus motif of TXY, which is catalysed by the upstream dual-specificity enzyme MAPKKs. Therefore, FLRS is identified as a MAPK gene in wheat.

Protein kinases mediate protein phosphorylation that plays a key role in most cellular activities by catalyzing the transfer of the gamma phosphate from nucleotide triphosphates to one or more amino acid residues in a protein substrate side chain, resulting in a conformational change affecting protein function. There are three broad classes for protein kinases, which are serine/threonine-protein kinases, tyrosin-protein kinases and dual specific protein kinases (e.g. MEK or MAPKK phosphorylates both Thr and Tyr on target proteins) (Hanks *et al.,* 1988). The function of protein kinases has been evolutionarily conserved from *E.coli* to human (Manning *et al.,* 2002). Protein kinases play a role in different cellular processes, such as division, proliferation, apoptosis, and differentiation (Manning *et al.,* 2002). In plants, protein kinases play a central role in signaling during pathogen recognition and the subsequent activation of plant defense mechanisms. Members of different kinase subfamilies, such as calcium-dependent protein kinases and MAP kinases, are involved. Here we studied possible involvement of FLR and FLRS in wheat defense response.

5.2 FLR and FLRS in plant defense responses to SA and FB1

There were no changes of FLR and FLRS transcript levels when wheat plants (Frontana and Roblin) were treated with SA and FB1, indicating that these two genes may not be involved in SA-mediated or FB1-induced pathways at transcrioptional level. However, ERK-like MAPKs (including FLRS) were post-translationally regulated during SA and FB1 treatments. ERK-type MAPKs in wheat resistant cultivar Frontana could be involved in the SA signaling pathway. Also, it indicates that ERK-type MAPKs play a role in wheat defense against FB1 toxin at post-translational level. These results are similar to the observation that SIPK was regulated strictly at the post-translational level by dual

phosphorylation of threonine and tyrosine residues in the conserved TEY motif (Zhang and Klessig, 1997). Recently, another similar result was reported where the wheat mitogen-activated protein kinases TaMPK3 and TaMPK6 were regulated at post-translational level by dual phosphorylation on Thr and Tyr residues (Rudd *et al.,* 2008).

The regulation at post-translational level but not at transcriptional level may be explained by the observation that the MAPK cascade components are regulated at multiple levels, which include transcriptional, translational and post-translational levels (Zhang *et al.,* 2000). Several studies have shown that the level of gene expression does not necessarily correlate with the protein levels in a cell (Gygi *et al.,* 1999). Many different variations of a protein can be generated by one or more covalent modifications of the polypeptide chain. Modifications can include phosphorylation, glycosylation, isoprenylation, acetylation, farnesylation and others to result in hundreds of variant amino acids (Roberts, 2002). Of these, protein phosphorylation represents a major control mechanism for protein activity in many organisms and in plants it appears to be the most predominant post-translational modification in response to pathogens. In the examination of proteins that were phosphorylated in *Arabidopsis* upon treatment with flagellin, a bacterial elicitor, only a few of the phosphoproteins were found to be regulated at the transcriptional level (Peck, 2003). Therefore, it is believed that analysis of protein levels and protein modification profiles, gives the best indication of the final players in a cellular response.

Actually, regulation at transcriptional, translational and post-translational levels are each important, and the relative contribution of each level to the overall response might vary. The tobacco WIPK gene (wound-induced protein kinases; which encodes a MAPK) is activated at several stages during the induction of cell death by fungal elicitins (Zhang *et al.,* 2000). *De novo* transcription and translation are necessary for the activation of the kinase activity and the onset of hypersensitive-response (HR)-like cell death. A fungal cell wall elicitor that does not induce cell death nonetheless induces WIPK mRNA and protein at similar levels to those observed with elicitins, but no corresponding increase in WIPK activity is detected (Zhang *et al.,* 2000). This indicates that post-translational control is also crucial in elicitin-induced cell death. In a study of a variety of stresses,

Ichimura *et al.* (2000) showed that low temperature, low humidity, hyper-osmolarity, touch and wounding induce rapid and transient activation of the *Arabidopsis* MAP kinases ATMPK4 and ATMPK6. Activation of ATMPK4 and ATMPK6 was associated with tyrosine phosphorylation but not with the amounts of mRNA or protein. In a recent wheat study, Rudd *et al.* (2008) demonstrated that the wheat mitogen-activated protein kinases TaMPK3 and TaMPK6 are differentially regulated at multiple levels during disease interactions. These studies have shown how the multiple levels of kinase regulation contribute to the final effectiveness of phosphorylation pathways.

As phosphorylation of ERK-type MAPKs at TEY motif was up-regulated by SA we further tested if SA-induced ERK phosphorylation may activate downstream components. Molina *et al.* (1999) showed that PR1.1 and PR1.2, considered as indicators of SAR in dicotyledonous plants, were unresponsive to SA and other SAR activators in wheat, a monocotyledonous species, but Lu *et al.* (2006) demonstrated that PR1.2 responded to SA whereas PR1.1 did not. While this result seems contradictory, the responsiveness could be age- and developmental stage-dependent (Lu *et al.,* 2006). We have examined whether wheat PR1.2 gene responds to SA, which is one of the activators of SAR. In order to know if SAR and PR1.2 are the downstream of ERK-type MAPK, we used ERK docking domain inhibitor or a general kinase inhibitor together with SA treatment. Our results showed that PR1.2 was unresponsive to SA in both wheat cv. Frontana and wheat cv. Roblin, which is consistent with the results previously published for wheat by Molina *et al.* (1999), but contrary to the observations in Lu *et al.* (2006). These differences may be a reflection of varietal differences, since we used different wheat cultivars from wheat cv. Kanzler used by Molina *et al.* (1999) and spring wheat cv. Laura used by Lu *et al.* (2006). During our experiment, the SA concentration in our work (100μM) was much lower than the one employed in Lu *et al.* (2006) (20mM) and in Molina *et al.* (1999) (3mM). Since exogenous SA did not have any effect on wheat PR1.2, we can't tell if SA-induced defense response is downstream of ERK-type MAPK in our wheat cultivars. Our results indicate that PR1.2 did not respond to ERK specific inhibitor, but PR1.2 transcript decreased when a general kinase inhibitor was applied, indicating that PR1.2 may not be downstream of ERK-type MAPK pathway but rather downstream of a different independent pathway in wheat.

5.3 FLR and FLRS are involved in wheat response to pathogens

It has been known that stress-induced transcription of genes for MAPKs play an important role in plant defense responses, so we would like to know if FLR and FLRS are involved in plant-pathogen interactions. Our results showed that FLR and FLRS expressions were significantly up-regulated during leaf rust and FHB challenges. Wheat near isogenic line Thatcher Lr16 was inoculated with the leaf rust pathogen, race BBB or race TJB. The wheat-leaf rust interaction follows the Avr gene-for-R gene model. FLR and FLRS were transcriptionally up-regulated upon Lr16-BBB incompatible interaction compared to Lr16 control and Lr16-TJB compatible interaction. Race BBB has the Avr gene that can be recognized by the R gene Lr16, leading to plant defense responses.

Many MAPKs investigated to date are involved in stress responses (Jonak *et al.*, 2002). In the genetic model plant *Arabidopsis*, MPK3, MPK4, and MPK6 are activated by a diverse set of stresses, including pathogens, osmotic, cold, and oxidative stresses (Ichimura *et al.,* 2000; Kovtun *et al.,* 2000; Nühse *et al.,* 2000; Petersen *et al.,* 2000; Desikan *et al.,* 2001; Asai *et al.,* 2002; Droillard *et al.,* 2002). A broad range of pathogens are recognized by plants through pathogen-associated molecular patterns (PAMPs), which are highly conserved molecular structures of pathogenic microbes (Nürnberger *et al.,* 2004). PAMP perception systems in plants and animals are mechanistically comparable, and PAMP recognition by receptors triggers nitric oxide, reactive oxygen species (ROS), and MAPK cascades that induce innate immune responses (Nürnberger *et al.,* 2004).

Many studies showed that MAPK pathway plays a role in defense response to pathogen attacks. MPK6-silenced *Arabidopsis* plants are compromised in resistance to different pathogens (Menke *et al.,* 2004). Dóczi *et al.* (2007) revealed that the *Arabidopsis* MKK3 pathway plays a role in pathogen defense. Also, Marcel *et al.* (2007) showed that mitogen-activated protein kinase kinase kinase (MAP3K) in barley contributes to partial resistance to barley leaf rust. Our recent study also showed that the mitogen-activated protein kinase kinase (MAPKK) tMEK2 from tomato (*Solanum lycopersicum*) is a key component in the defense pathways against pathogen attacks, because tMEK2-transgenic

wheat (*Triticum aestivum*) has partial resistance to wheat leaf rust (caused by *Puccinia triticina*) (Fan *et al.,* 2009). FHB-resistant cultivar is the most cost-effective method to control the FHB disease (Yang *et al.*, 2006). These results indicate that MAPK pathway plays a role in regulating resistance in plant-pathogen interactions. Potentially it is possible to enhance disease resistance by activating FLR and FLRS in wheat.

5.4 SA and FB1 induce cell death in wheat

Programmed cell death is recognized as an essential physiological and genetic process occurring during plant development and in response to biotic and abiotic stresses (Beers and McDowell, 2001). Many host responses precede the HR, including proteolysis, changes in ion fluxes, production of reactive oxygen and nitrogen species, and activation of MAPK cascades. MAPKs are involved in regulation of cell death (Pozo *et al.*, 2004; Yang *et al.*, 2001; Zhang and Liu, 2001). Recently, Mekk1 homozygous knockout plants show a severe dwarfism, accumulate high amounts of reactive oxygen species (ROS), and develop local lesions reminiscent of PCD (Ichimura *et al.*, 2006; Nakagami *et al.*, 2006). The results provide the evidence that MAPK pathway plays a role in cell death regulation.

In this project, FLR and FLRS have been identified as MAPK pathway components, and FLRS has been identified as ERK-type MAPK. Our result indicated that ERK-type MAPKs in wheat resistant cultivar could be involved in the SA signaling pathway, and ERK-type MAPKs play a role in wheat defense against FB1 toxin at posttranslational level. Furthermore, FLR and FLRS have been suggested to be involved in defense responses to leaf rust and Fusarium head blight challenge in wheat. Therefore, it is believed that FLR and FLRS play important roles in plant defense to stresses. This led us to clone these two genes and try to demonstrate if FLR and FLRS play a regulatory role when overexpressed or silenced in wheat. Also, if FLR or FLRS silencing abrogates the programmed cell death caused by SA or FB1, it may suggest that wheat FLR and FLRS are involved in cell death regulation. Therefore, we have to verify that SA and FB1 can induce cell death in wheat first.

We have analysed the SA and FB1 induced cell death in wheat FHB resistant and susceptible cultivars, Frontana and Roblin. In both of these cultivars, cell death occurred when wheat leaves were treated with SA or FB1. Our result also suggests that cell death in wheat is associated with concurrent accumulation of ROS. Furthermore, our result shows that SA and FB1 treatments triggered DNA smearing in wheat, suggesting that wheat leaves mainly undergo necrosis instead of apoptosis (or both) with SA and FB1 treatment. The mechanisms underlining this DNA smearing should be further studied for a better understanding of the SA and FB1 induced cell death in wheat leaves.

Our results are consistent with previous studies, which have shown that cell death, SA, FB1, ROS, and MAPK cascades are strongly interconnected. For examples, addition of SA to either soybean suspension cultures or *Arabidopsis lsd1* mutants accelerated cell death (Dietrich *et al.,* 1994; Shirasu *et al.,* 1997). The characterization of two other *Arabidopsis* mutants *acd* and *lsd* which activate PCD also supported the crucial role of SA in cell death processes, because both of these two mutants have constitutively high SA levels (Rate *et al.,* 1999; Brodersen *et al.,* 2002). Moreover, exogenous application of SA in *acd11/eds5-1 Arabidopsis* mutants also accelerated PCD (Brodersen *et al.,* 2005). ROS play a role in programmed cell death in plants, and MAPK cascades are key players in ROS signaling. MAPK signaling pathways are not only induced by ROS but can also regulate ROS production. Phenotypic and molecular analysis revealed that the MAPK kinases MKK1 and MKK2 are part of a cascade, regulating ROS and SA accumulation (Pitzschke *et al.*, 2009). Moreover, Liu *et al.* (2007) indicated that the MAPK cascade actively blocks carbon fixation, leading to an excess of excitation energy in illuminated plants and consequently accumulation of ROS. The sustained generation of ROS cannot be compensated for by the action of antioxidant enzymes, and, after depletion of the antioxidant pool, leads to cell death. Therefore, ROS plays an intermediary role between MAPK cascade and PCD. FB1 induces apoptosis-like programmed cell death (PCD) in both plants and animals (Asai *et al.*, 2000). In *Arabidopsis*, FB1 treatment initiated nuclear DNA fragmentation preceding the loss of membrane integrity, which resembles apoptosis typically associated with PCD in animal cells (Stone *et al.*, 2000). Asai *et al.* (2000) have proposed a model for cell death signaling in *Arabidopsis* protoplasts induced by FB1. They demonstrated that FB1-elicited PCD requires SA, jasmonic acid (JA) and

ethylene (ET)-dependent signaling pathways as well as one or more unknown factors activated by FB1.

5.5 Conclusion and future work

In this study, we have identified wheat FLR and FLRS genes, which are MAPKK kinase and MAP kinase respectively, and FLRS has been further identified as an ERK-type MAPK. At transcriptional level, FLR and FLRS may not be involved in SA-mediated pathway, and they may not play a role in wheat response to FB1. However, ERK-type MAPKs (including FLRS) in wheat FHB resistant cultivar Frontana could be involved in the SA signaling pathway, and ERK-type MAPKs play a role in wheat defense against FB1 toxin at post-translational level. PR1.2 may not be downstream of ERK-type MAPK pathway in wheat, and PR1.2 was unresponsive to SA treatment. Furthermore, FLR and FLRS have been suggested to be involved in defense responses to leaf rust and Fusarium head blight challenge in wheat. FLR and FLRS cloning work is in progress now. SA and FB1 treatments induced cell death in wheat, which appeared to be necrosis and associated with concurrent accumulation of ROS.

Our work has indicated that FLRS, as an ERK-type MAPK, is involved in wheat responses to SA and FB1 at post-translational level. To further delineate the function of FLR and FLRS in the future, it is probably necessary to overexpress them in wheat plants. Transgenic plants will be tested for their responses to biotic and abiotic stresses and for their function in plant growth and development. RNAi gene silencing approach (Smith *et al.*, 2000) can be employed to silence FLR or FLRS in wheat, in order to see if they are involved in cell death regulation.

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