IDENTIFICATION AND CHARACTERIZATION OF *F. GRAMINEARUM*-INDUCED ESTS FROM WHEAT SPIKES

By

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NOMENCLATURE

AFLP	amplified fragment length polymorphism
AUDPC	area under disease progress curve
BLAST-X	basic local alignment search tool-translated
bp	base pair
cDNA	complementary deoxyribonucleic acid
DD	differential display
DNA	deoxyribonucleic acid
DIMBOA	2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one
DON	deoxynivalenol
COMT	caffeic acid O-methyltransferase
dNTP	deoxy nucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid
EST	expressed sequence tag
FHB	Fusarium head blight
h	hours
hai	hours after inoculation
JA	jasmonic acid
min	minute
MJ	methyl jasmonate

mRNA	messenger RNA
NIL	near isogenic line
OSU	Oklahoma State University
PAL	phenylalanine ammonia lyase
PCR	polymerase chain reaction
PR	pathogenesis-related
QTL	quantitative trait locus
RAPD	random amplified polymorphic DNA
RDA	representational difference analysis
RNA	ribonucleic acid
RFLP	restriction fragment length polymorphism
RIL	recombinant inbreed line
ROS	reactive oxygen species
RT-PCR	real time-polymerase chain reaction
SA	salicylic acid
sec	seconds
SSC	sodium chloride-sodium citrate
SSH	suppression subtractive hybridization
SSR	simple sequence repeats
STS	sequence tagged sites
TAE	tris-acetic acid-EDTA
TLP	thaumatin-like protein
USDA	United States Department of Agriculture

CHAPTER I

A LITERATURE REVIEW

Fusarium Head Blight

The pathogen

Several species of *Fusarium* have the ability to cause Fusarium head blight (FHB). *Fusarium graminearum* Schwabe (teleomorph=*Gibberella zeae*) has been the most predominant species responsible for FHB in North and South America, Central and Southern Europe, Japan and China (Stack, 1999). *F. culmorum* and *F. avenaceum* are also causal species in some parts of Northern Europe (Parry et al. 1995). *F. graminearum* is a soil- and residue-borne fungal pathogen that is capable of producing both asexual spores (conidia) and sexual spores (ascospores). Although both types of spores are capable of infecting susceptible heads of wheat at anthesis, ascospores are the major source of inocula for initial infection in nature. Ascospores are released to air by force and transported to wheat spikes by wind or rain splashing.

Disease development

Wheat at flowering stage is the most susceptible stage at which infection occurs although it may also occur until the soft dough stage of kernel development (Schroeder and Christensen, 1963). According to several reports (Dickson et al. 1921; Pugh et al. 1993 and Tu, 1953), entry of the pathogen occurs through protruding anthers, then extends to glumes and rachis. However, Pritsch et al. (2000) reported that the penetration of the pathogen was through the stomata after studying *F. graminearum* development in spray-inoculated wheat spike. A detailed investigation on the infection process revealed that macroconidia germination on the apical surface of the glume occurred at 6-12 hours after inoculation (hai). The hyphae started to make contact with the stomata at 12-24 hai,

followed by thickening of the hyphae at 36-48 hai and branching along stomatal rows 48-76 hai.

A study conducted by Kang and Buchenauer (2000) using the single floret inoculation method to infect wheat heads with *F. culmorum* revealed that the pathogen directly enters the top part of the ovary, inner lemma and inner palea 36 to 48 hai. In addition, there is no difference in the infection process and initial spread of infection between resistant and susceptible varieties but slower pathogen development was observed in the resistant varieties Arina and Frontana. In Sumai 3, histological studies showed a delay in fungal development as well as a delay in colonization of vascular bundles in the rachis by several days compared to susceptible varieties (Ribichich et al. 2000).

Environmental conditions that favor disease development are high temperature (22-25ºC) and high humidity. Glumes of infected spikelets first appear to have light brown water-soaked spots. As the disease spreads from an infected spikelet to neighboring spikelets through the rachis, the vascular tissues in the rachis becomes clogged and spikes become prematurely ripe (Adams, 1921; Dickson et al. 1921; Pugh et al. 1933). Bleaching of any part (usually the upper half) or all of the head is the most obvious symptom of this disease. Clogging of the vascular tissues will lead to shortage of water and nutrients and infected florets will not be able to produce grain or if any, poorly–filled grain (Bai and Shaner, 2004). Severe infection at a very early stage significantly reduces grain yield and quality. The timing of infection, abundance of primary inoculum, temperature and humidity determines the severity of FHB. If high

moisture is available, pink mycelium grows on spikes and spikes appear to be pink in color, therefore, FHB is also known as pink mold disease in Japan and China.

Toxin production

In FHB infected grain, *F. graminearum* produces a toxin called deoxynivalenol (DON), a trichothecene. There is significant variation of DON levels among wheat varieties. Compared to susceptible varieties, DON levels are lower in resistant varieties (Mirocha et al. 1994) and the amount of DON in susceptible varieties may be up to eight times higher than in resistant varieties (Miller et al. 1985). Among 116 varieties and breeding lines surveyed by Bai et al. (2001) DON levels ranged from trace amounts to 283 mg/kg in greenhouse tests. In addition, the amount of DON is proportional to fungal biomass.

Effects of DON include refusal of animals to feed on infected grain (Xu and Chen, 1993) and diarrhea in both human and animals after ingestion of high levels of this toxin. In addition, dizziness, headache, vomiting and fever may occur in humans. For health reasons, USA, Canada and some European countries have set the maximum acceptable amount of DON in wheat grains at 0.5-2 mg/kg.

Trichothecene belongs to the sesquiterpenoid secondary metabolites family and this toxin inhibits protein synthesis in eukaryotes (Kimura et al. 1998). In addition, they bind to the 60S ribosomal subunit and prevent polypeptide chain initiation or elongation. The effect of this toxin on the virulence of the pathogen was investigated using Tri5 mutants. The Tri5 gene encodes for trichothecene synthase, the enzyme that catalyzes the first step in the trichothecene biosynthetic pathway. Since Tri5 mutants cannot produce trichothecene synthase, neither is DON (the end product of the biosynthetic pathway)

produced. Some researches indicated that DON might contribute to the virulence of the pathogen. Studies showed that compared to the wild type, Tri5 mutants caused significantly fewer disease symptoms in wheat (Proctor et al. 1995; Desjardins et al. 1996; Nicholson et al. 1998). Bai et al. (2001), moreover, reported that the ability of both wild type and Tri5 mutant to cause initial infection was the same in both field and greenhouse conditions. However, disease spread was only observed in plants infected with DON-producing strain (Tri5) implying that DON plays an important role in spread of FHB disease.

Proctor et al. (1997) reported that Tri5 revertants generated by transformation-mediated complementation had virulence comparable to the wild type. However in another study, Desjardins et al. (2000) were not able to restore the virulence of Tri5 complemented mutants to wild type levels because the transformation-mediated gene process affected not just the Tri5 gene but other loci as well.

Sources of resistance

One of the most practical and effective means of controlling scab is to grow FHB resistant varieties. Out of 17,000 wheat accessions screened in China, 32 were reported to be highly resistant to FHB (CWSCG, 1984). Some of these accessions were used as parents in breeding programs but efforts to incorporate FHB resistance to elite lines have been unsuccessful because other undesirable agronomic traits, such as small heads and late maturity, were also transferred. In breeding programs worldwide, the most widely utilized source of scab resistance is Sumai 3 and its derivative, Ning 7840 (Wilcoxon, 1993). Sumai 3 is a transgressive segregation progeny that originated from a cross of moderately susceptible Italian wheat variety Funo and Chinese landrace Taiwanxioamai

(Liu and Wang, 1990). Chinese breeders have used Sumai 3 for at least 20 years (Liu, 1984) and its resistance has not yet been broken. Ning 7840 has also been widely used in China because in addition to having the same level of FHB resistance as Sumai 3, it has also been shown to be resistant against rust, powdery mildew and has better agronomic traits. The CIMMYT breeding program has also been using Sumai 3 as well as lines with Sumai 3 parentage (Shanghai and Wuhan series) for more than 20 years in breeding for FHB resistance in wheat. Sumai 3 has good combining ability for FHB resistance as well as yield traits (Bai et al. 2003a; Ban, 2000a; Gilchrist et al. 2000; Lu et al. 2001). The US Uniform Regional Scab Nursery houses a collection of FHB resistant lines from major breeding programs, and FHB tests conducted from 1995-2000 showed that ca. 60% of the resistant lines had Sumai 3 in their pedigrees (Garvin and Anderson, 2002).

Other sources of FHB resistance are Chokwang, originally from Korea, (Shaner and Buechley, 2001) and Fundulea 201R from Romania (Shen et al. 2003). These two are not related to Sumai 3 and may carry different FHB resistance gene(s). In Fundulea 201R, this was supported by the fact that its major QTL did not map to the same position as with Sumai 3. Japanese wheat accessions such as Schinchunaga, Nobeo kabuozu and Nyu Bai have been identified to have good levels of FHB resistance (Ban, 2000a; Ban, 2000b) but like other FHB resistant landraces in China, their agronomic traits are not as good as that of Sumai 3 (Ban, 2001). Freedom and Ernie were reported to be FHB resistant under field conditions and have been used by some breeders in the US. Both varieties do not have the 3BS QTL for FHB resistance (Bai et al. 2003b). Frontana and Encruzilhada are cultivars from Brazil that have FHB resistance (Ban, 2001; Desjardins et al. 1996; Masterhazy, 1997). Although Frontana did not have resistance to spread of

infection (discussed below) based on greenhouse test (Bai, pers comm.), field test results show that it had low disease incidence (Singh et al. 1995). Other sources of scab resistance have been reported from several countries (Dubin et al. 1997) but they are not widely used in breeding programs.

Types of resistance

Resistance to FHB is classified into five types: (I) resistance to initial infection; (II) resistance to spread of infection; (III) resistance to kernel infection; (IV) tolerance and (V) resistance to toxins (Schroeder and Christensen, 1963; Wang and Miller, 1988; Mesterhazy, 1995).

Resistance to initial infection (type I) is evaluated by spraying spikes evenly with conidiospores (50000 spores/ml) at 50% anthesis (Rudd et al. 2001). Another round of spraying is usually done a week later to infect plants that were not at anthesis during the first spraying. The number of infected spikelets is counted 21 days post-inoculation and % infected spike is determined. When disease evaluation involves many plants, estimation of the proportion of spikes with FHB is usually done by visual inspection (Bai and Shaner, 2004). In some breeding programs, observations are made at regular 3-5 day intervals and summarized as area under the disease progress curve (AUDPC). The AUDPC reflects the increase in disease severity as a result of the invasion of spike by the pathogen after inoculation and not due to secondary infection.

Type II resistance is the most stable form of resistance and has been consistent across environments (Bai and Shaner, 1994). It is measured by the single-floret inoculation method. Conidiospores (~1000 spores) are inoculated to a centrally located floret and spread of infection within a spike is estimated by counting the number of

blighted spikelets after some period of time (Bai and Shaner, 1996). Another way of assessing the spread of infection is by visual estimation of the proportion of spikes with blight symptoms by comparing to checks with some degree of type II resistance. Blighted spikes in susceptible varieties may be observed as early as 10 days post inoculation.

It is difficult to distinguish type I from type II resistance under field conditions because environmental conditions are hard to control (Bai and Shaner, 2004). When evaluating plants for type I resistance, it is assumed that each spikelet received equal amounts of inoculum. However, it is difficult to tell if each spikelet received equal amounts of inoculum and some spikelets may be missed during spraying. If the plant has no type II resistance, disease will spread and resistance or susceptibility to type I is masked (Shaner, 2002). For accurate measurement of type I resistance, plants must have resistance to spread of infection (Rudd et al, 2001). In addition, the inoculation should be done during anthesis, or else what may appear to be type I resistance could simply be disease escape (Bai and Shaner, 2004).

In a line with a high level of type II resistance, infection will be observed only on the initially infected floret, but if extremely favorable environmental conditions for infection of the pathogen coincides with anthesis and a lot of inocula are present in the field, infection at multiple sites of the spike happens frequently (Shaner, 2002).

Type III resistance is resistance to kernel infection and is measured as the percentage of infected kernels (Bai and Shaner, 2004). Reduction in the number of kernels, weight and estimation of kernel damage by visual infection are parameters used to evaluate Type III resistance (Rudd et al. 2001). Testing for this form of resistance is complicated because if point inoculation is done and the plant has Type II resistance, the

pathogen will not spread and will have no chance to infect the kernels and the plant would seem resistant. Spray inoculation is also not appropriate because if Type I resistance is present then resistance or susceptibility to kernel infection is masked (Shaner, 2002).

Type IV resistance refers to tolerance. This means that plant yield is not significantly affected even when FHB symptoms are severe and comparable to that of the susceptible plant. If a plant is tolerant, reduction in grain yield and quality will be little, but such observations can also be due to the pathogen not being able to infect the kernels (Shaner, 2002). Type III resistance may confound measurement of Type IV resistance.

Type V resistance is decomposition or non-accumulation of mycotoxins. Plants having this type of resistance may have found ways to inhibit DON production or detoxify the DON produced by the pathogen (Bai and Shaner, 2004). Non-accumulation or low accumulation of DON could be due to accumulation of DON in tissue other than kernels or low DON production by the fungus.

The amount of DON present in harvested grain is assayed by using a fluorometric quantitation method (Bai et al. 2001). DON was proposed as a virulence factor and is involved in initial infection by *F. graminearum*. The level of DON production is related to the biomass of the fungus. The greater the number of infected seeds, the greater the amount of DON detected. Low DON content of harvested grain could result from fewer infected kernels. Fewer infected kernels may be due to the plant having at least one of either Type I or II resistance (Bai and Shaner, 2004). Genotypes with Type I or II resistance would have fewer infected kernels and lower DON levels compared to susceptible plants. Low DON readings in infected kernels of a susceptible plant may be

the result of light shriveled infected grain getting threshed out because of their weight (underestimation of DON). On the other hand, infection of a resistant plant could still lead to partial or full grain filling. These grains would be heavier and will not be blown away during threshing and resistant plants would seem to have higher DON content. Bai and Shaner (2004) suggested that spikes be hand threshed for accurate measurement of Type V resistance.

Inheritance of wheat FHB resistance

There have been conflicting reports as to the number of genes that control scab resistance. Nakagawa (1955) suggested that at least three genes govern FHB while Yu (1982) suggested more than three. Three or more genes were reported to condition FHB resistance in the spring wheat variety Frontana (Singh et al. 1995). In contrast, work by Van Ginkel et al. (1996) showed that Frontana and Ning 7840 were both controlled by two dominant genes. In several other studies, Sumai 3 was reported to have two to three genes for FHB resistance (Yao et al. 1997; Bai et al. 1994; Bai et al. 2000). The variation in the estimated number of genes governing FHB resistance in wheat could be due to the polygenic control of FHB resistance, effect of different genetic background, different types of resistance evaluated, genotype and environment interactions, heterogeneous sources of a resistant parent or inoculation techniques used in different studies (Kolb et al. 2001).

Markers linked to FHB resistance

Molecular markers are landmarks that serve to identify the location of a particular gene. In wheat, RAPD, RFLP, AFLP, SSR and STS markers linked to FHB have been

reported, and most of them have been successfully mapped to a particular chromosome.

RAPD markers linked to two scab resistance loci were found using two different mapping populations: recombinant inbred lines (RILs) from a Ning 7840/Clark population (Bai, 1995) and from a Fukuhokomugi/Oligo Culm double haploid population (Ban, 1997). In addition, Ban and Suenaga (1997; 1998) mapped RAPD markers into chromosome 5AL using two double haploid lines with Sumai 3 as one of the parents.

Waldron et al. (1999) found RFLP markers linked to Sumai 3 scab resistance in chromosome arms 3BS and 6BS while those associated with resistance derived from the Stoa variety were in 2AL and 4BL. However in a line derived from Sumai 3, ND2603, FHB resistance QTL was mapped in chromosome arm 3AL (Anderson et al. 1998). The finding that a FHB resistance QTL of Sumai 3 was located in the short arm of chromosome 3B was further supported by identification of several SSR markers (Buerstmayr et al. 2002; Chen et al. 2000; Gupta et al. 2000; Zhou et al. 2002). Moreover, Bai et al. (1999) found 11 AFLP markers linked to scab resistance in Sumai 3. All the markers mapped to a single linkage group (chromosome arm 3BS) and one marker, a major QTL for scab resistance, explained up to 53% of the phenotypic variation and also associated with low DON accumulation in infected kernels. Further study indicated that the AFLP linkage group harboring the major QTL also belongs to 3BS (Zhou et al. 2001). To make markers technically less demanding and easier to be used in marker-assisted selection, Guo et al. (2002) converted an AFLP marker for the major QTL on 3BS into an STS marker. Other locations of FHB resistance genes were reported to be in 2AS and 7BS (Gupta et al. 2000), 6BL (Anderson et al. 1998), 5A and 1B (Buerstmayr et al. 2002).

Molecular mapping of the major QTL for FHB resistance in Ning 7840 (Zhou et al. 2002) showed that its location is the same as that of Sumai 3 (3BS). In addition, QTLs on 2BL and 2AS were found to enhance the resistance conferred by major QTL on 3BS.

Biochemical and molecular basis of FHB resistance

The biochemical basis of FHB resistance has also been studied. Differences in superoxide dismutase, catalase, phenylalanine ammonia lyase and ascorbic acid oxidase activities between resistant and susceptible varieties have also been reported (Chen et al. 1997; Lu et al. 2001). There was an increase in peroxidase activity in infected spikes in both resistant and susceptible varieties but the enzyme acitivity was observed for a longer period of time in the resistant variety (16 days) than in the susceptible variety (8 days) (Xu et al. 1991). Chen et al. (1997) observed that superoxide dismutase activity in infected spikes was significantly higher by 200-300 U/gfw in resistant plants than in susceptible plants. Other chemicals such as choline were present at only half the levels in resistant plants (Li and Wu, 1994). The phenolic compound chlorogenic acid was found to be higher in Nannong 824 (susceptible) than Sumai 3 (resistant).

Traditionally, genes were isolated from cDNA libraries – a collection of genes expressed in a particular tissue at a particular time or stage of development. Often, only a part of the full-length gene is isolated and the partial sequence is called an expressed sequence tag (EST). Even though they only contain part of the gene, they are of sufficient length to 'tag' the gene they represent. ESTs generated from a library combined with database mining have been successfully used for large-scale gene identification in both plant and animal species (Adam et al. 1991; Sasaki et al. 1994). Using this technique, Li et al. (2001) reported the isolation of chitinases and β-1,3-glucanases from *F.*

graminearum- infected Sumai 3 spikes using rice chitinase and barley chitinase and β-1,3-glucanase as probes. Expression analysis revealed that transcript levels were the same for Sumai 3 and its susceptible mutant but differ in time of expression. Peak expression (relative to mock-inoculated control) in Sumai 3 was at 24 hai or earlier, while in the mutant high transcript accumulation occurred at 48 hai or later indicating a slower defense response in the susceptible mutant. Gene expression levels were very low in uninoculated, moderate in mungbean broth inoculated and highest in scab-infected plants.

More recently, nine defense-related genes, three stress-induced and three R-genes were isolated by Fellers et al. (2002) from a wheat cDNA library constructed from Sumai 3 spikes infected with *F. graminearum* for 24 hai. In another study, spray-inoculated Sumai 3 spikes sampled at 0, 6, 12, 24, 36, 48 hai were combined and used to construct a cDNA library (Kruger et al. 2002). Among the genes isolated were defense-related ESTs such as PR-1, β-13-glucanase, chitinase and thaumatin-like proteins (TLPs). Other genes identified from the library were involved in the phenylpropanoid pathway, oxygen metabolism, lipid transfer and cell protection.

Anti-fungal genes

Three anti-fungal genes namely, *TRI101, GAFP* and *Wch2*, were cloned and used for enhancing wheat scab resistance through transgenic approach. The *TRI101* gene is 1356 base pair (bp) long and codes for an enzyme that catalyzes a specific O-acetylation at the C-3 position of the tricothecene ring rendering it non-toxic (Kimura et al. 1998). It was cloned from a *Fusarium gramineum* cDNA library made from mycelia grown in the presence of the trichothecene T-2 toxin. The successful transformation of the *TRI101*

gene into the cultivar Bobwhite was reported by Okubara et al. (2002) and one transgenic line showed significantly less disease compared to untransformed plants.

A purified GAFP (*Gastrodia* antifungal protein) from the traditional Chinese medicinal herb, *G. elata*, was found to have strong antifungal activity to *Gibberella zeae* and other fungi. In 2000, Wang et al. isolated a 534 bp *GAFP* gene encoding 178 amino acids from a *G. elata* wheat cDNA library. The molecular weight of this protein is 14 kDa and the deduced amino acid sequence was found to have homology with mannose-binding lectins. Its effective concentration to a broad spectrum of fungi was found to be at 60 μg/ml. It effectively inhibits both spore germination and hyphal growth of *G. zeae in vitro* but exactly how it was able to do this is still unknown.

Chitinases are enzymes that hydrolyze chitins, an important cell wall component of several genera of fungi (Bartnicki-Garcia, 1968). Such hydrolysis would inhibit growth of *Fusarium* and DON production. Zhong et al. (2000) cloned a class I chitinase gene from a wheat genomic library. This gene, designated *Wch2*, encodes for 317 amino acid residues that has sequence similarity of 73-83% and 68-74% to chitinases from monocots and dicots, respectively. Another group of pathogenesis-related protein is the thaumatin-like proteins (TLPs). TLPs disrupt the cell membrane of the pathogen thereby preventing fungal growth and toxin production. Rebmann et al. (1991) cloned a TLP gene from a cDNA library made from wheat inoculated with *Erysiphe graminis* f. sp. hordei. This gene, called *PWIR2*, translates to 173 amino acids and has a molecular weight of about 17 kDa.

Chen and co-workers (1999) obtained 34 trangenic plants with high levels of TLP gene expression and were able to bioassay plants from T_1, T_2 and T_3 generations using *F.*

graminearum conidia. Compared to untransformed wheat, the transgenic plants had significantly delayed expression of FHB symptoms.

Methods for Studying Differential Gene Expression

Differential display RT-PCR (DDRT-PCR)

In this method, total RNA from two populations (e.g. resistant and susceptible plant) are isolated and mRNA is reverse transcribed using 3 different oligo(dT) anchored primers (5'-T_nTTTTA-3', 5'-T_nTTTTC-3' and 5'-T_nTTTTG-3') to obtain 3 cDNA subpopulations (Liang and Pardee, 1992). Homogenous initiation of cDNA synthesis at the beginning of the poly (A) tail of all mRNAs is made possible by the use of anchored primers. The subpopulations are amplified and labeled with radioactive nucleotides via PCR using arbitrary 13-mers and anchored primers. PCR fragments are separated and visualized by autoradiography. Differentially expressed ESTs are seen as bands present in one population and absent in the other or bands of different intensities. Bands corresponding to differentially expressed cDNAs are excised from the gel and ligated to a T/A cloning vector. The advantage of this method is that it is technically simpler and one can see the products/ESTs before cloning. Its disadvantages are that at least 240 primer combinations must be used to achieve 95% coverage of the transcriptome (Liang and Pardee, 1992) and it is prone to up to more than 50% false positives. In addition, ESTs obtained from DDRT- PCR are short and getting full-length gene will be difficult.

Xing et al. (2000) used DDRT-PCR to identify differentially expressed genes between scab-infected Sumai 3 and Wheaton. The following gene expression patterns were reported: (1) constitutive expression in either variety, (2) induced expression in both

varieties, (3) induced expression only in fungal-infected Wheaton, and (4) induced expression only in fungal-infected Sumai 3. Three ESTs only expressed in scab–inoculated Sumai 3 (having similarity to mRNA for polypeptide elongation factor 1-β, pathogen-induced sorghum bicolor cDNA and wheat gene for chloroplast ATP synthase CF-O subunit 1 and 2) were proposed as defense–related genes.

Representational difference analysis (RDA)

This technique was developed by Lisitsyn et al. (1993) to identify differences between two complex genomes. A modification of this technique, cDNA RDA, uses mRNA as the starting material instead of DNA. Messenger RNA from tester (contains genes of interest) and driver populations ("baseline" population) are separately reverse-transcribed to cDNA and double stranded cDNA is digested with *DpnII*. Oligonucleotide adapters (R-adapters) are ligated to digestion products and PCR amplified. R-adapters are digested away and J-adapters are added to tester population. Tester and driver populations are then mixed and subjected to rounds of subtraction and amplification. Common sequences between the 2 populations are not amplified while unique sequences are exponentially amplified because of the J-adapters. A very important consideration when using this technique is that amplification must be kept in a linear range. If amplification is not kept in linear range, relative proportions of cDNAs will not be maintained and some genes will be over represented. One disadvantage of this technique is that it requires two rounds of hybridization. Its advantages are that only small amounts of RNA are needed and it generates less false positives compared with DDRT-PCR.

Suppression subtractive hybridization (SSH)

More recently, Diatchenko et al. (1996) developed a technique called suppression subtractive hybridization (SSH) to isolate differentially expressed cDNAs. Messenger from tester and driver populations are reverse-transcribed to cDNA. Double-stranded-DNA is then digested with *RsaI*. The tester population is divided into two portions and each is ligated to a different adaptor. No adaptors are ligated to the driver population. The cDNAs from the tester population are hybridized to an excess of cDNAs from the driver population and then unhybridized cDNAs (differentially expressed) are selectively amplified. Only the target (unique) cDNA fragments will have 2 kinds of adaptors and will be selectively amplified while non-target DNA amplification is suppressed. The advantage of this technique is that it combines normalization and subtraction in one step. The abundance of cDNAs in the target population is equalized in the normalization step by standard hybridization kinetics while sequences that are common between the tester and driver populations are subtracted out in the subtraction step. Moreover, SSH makes possible the isolation of transcripts of low or medium abundance. This technique requires little amounts of RNA but is technically more complex and expensive than DD-RT PCR and RDA.

In rice, Xiong et al. (2001) successfully identified 34 genes that were induced by jasmonic acid, benzothiadiazole and/or blast infection. Fifteen of the genes were homologous to some plant genes with known function, including defense-related and genes involved in signal transduction.

cDNA microarrays

This technique, introduced by Schena et al. in 1995, is a high throughput method for monitoring gene expression. PCR products from cDNA libraries or oligonucleotides are robotically printed onto chemically coated glass slides. Experimental and control cDNAs are labeled with two different Cy dyes and simultaneously hybridized to the arrays. The labeled RNA/DNA corresponding to the gene of interest will bind to its complementary DNA arrayed on the slide. If a particular gene's mRNA is more abundant in one population, then more of it will bind to the spot representing the gene and the color of the dye it is tagged with will predominate. Differential gene expression is analyzed using a confocal scanner that distinguishes Cy-3 and Cy-5 probes from each other. The scanner uses lasers to excite the probe. Since emission of energy is linear, the signal detected is directly proportional to the amount of fluorescence-labeled probe. Background signal is subtracted, data is normalized and the ratio of the fluorescence intensity between experimental and control cDNA is calculated to determine differentially expressed genes.

DNA microarrays allow the simultaneous analysis of thousands of genes in one experiment (Freeman et al. 2000; Hedge et al. 2000). It enables the detection and identification of up- or down-regulated genes. A lot of the microarray work has been reported for the model plant Arabidopsis. Schenk et al. (2000) used microarrays to analyze the expression profile of 2,375 genes from a cDNA library made from Arabidopsis that had been infected with *Alternaria brassicicola* or treated with salicylic acid (SA), methyl jasmonate (MJ) or ethylene. Findings revealed that 168 genes were up-regulated by at least 2.5 fold, while 39 genes were down-regulated in the pathogen-

induced library. Treatment with defense-related signaling molecules showed 192, 221 and 55 genes had at least 2.5 fold increase in expression after induction with SA, MJ and ethylene, respectively. In addition, 169 genes were co-regulated, implying that the plant defense responses involved coordination among signaling pathways. Furthermore, global analysis of gene expression profile of 8200 high-density arrays in *Arabidopsis thaliana* revealed that genes involved in plant defense (genes encoding for signaling molecules, cell wall modification enzymes, secondary metabolism, osmotic stress and heat–shock) were activated or repressed as a response to wounding (Cheong et al. 2002). Using Affymetrix gene chips, Puthoff et al. (2003) identified 128 genes with altered steady-state mRNA levels in response to cyst nematode parasitism. Schenk et al. (2003) studied the systemic gene expression in Arabidopsis challenged with *A. brassicicola* by comparing expression profiles in local and distal tissues. Twenty-five genes involved in plant defense, ß-oxidation pathway of fatty acids, cellular housekeeping, signal transduction and cell wall synthesis were found to be significantly up-regulated in distal tissues. Ten genes were identified as down-regulated by at least 2-fold. Their results show that local infection activates a signaling process that makes distal tissues prepared for subsequent infections. Ramonell et al. (2002) used an Arabidopsis array with 2375 ESTs to study transcriptional responses to chitin. Based on a 3-fold cut-off level of gene expression, 61 genes were reported to be differentially expressed between treated and control plants. Change in gene expression was observed 10 min after treatment with chitin and transcript accumulation was found the highest (25 genes) at 30 min after inoculation.

Analysis of gene expression profiles of 1400 cDNAs arrayed on glass slides conducted by Ruan et al. (1998) identified genes differentially expressed between leaf

and root and between leaf and flower. This demonstrates the usefulness of microarrays in identifying genes that are differentially expressed between plant organs.

A number of studies on the use of microarray to characterize the expression profile of plant genes involved in abiotic stress have been reported (Cushman and Bohnert, 2000; Bohnert et al. 2001; Kawasaki et al. 2001). Seki et al. (2002) monitored the transcriptional responses of 7,000 Arabidopsis genes after ABA-, drought-, cold-, and salt-stress treatments and observed 245, 54, 299 and 213 up-regulated genes, respectively. In addition, greater crosstalk between signaling pathways for drought and ABA responses was reported compared to ABA and cold responses. In a parallel study in rice, more crosstalk between drought-, ABA- and salt-stress had been observed than between cold- and ABA-stresses or cold- and salt-stress. Fifteen genes were found to respond to all four treatments (Rabbani et al. 2003). Both studies show that microarrays can be used to monitor crosstalk between signaling responses.

A tomato cDNA array was used by Frick et al. (2002) to study gene expression changes in response to fusicoccin and results show the inverse relationship between pathogenesis-related and wound-response genes. Defense response genes were induced while wound-response genes were down-regulated.

Microarray analysis has also been used to identify genes that are involved in activation or repression of major pathways. Scheideler et al. (2002) monitored the expression profiles of 13,000 ESTs obtained from *P. syringae* pv. *tomato*-infected *A. thaliana* and reported that genes involved in glyoxylate metabolism may be involved in plant defense. Other findings were that about 650 genes were induced as early as 10 min after infection and ~2000 genes were up- or down-regulated by 7 hours after inoculation

of the pathogen. Results of cDNA microarray analysis conducted in wild rice showed that genes involved in glycolysis, Krebs cycle and pentose phosphate pathway are up-regulated in response to fungal (*Magnaporthe grisea*) stress (Shim et al. 2004).

Literature on gene expression in response to *F. graminearum*-stress is limited. We hypothesize that resistance to FHB probably involves an integrated set of genes. The molecular mechanism of wheat-*F. graminearum* interaction must be well understood to facilitate biotechnology-assisted development of FHB-resistant varieties. Genome-wide analysis of gene expression in response to FHB infection must be done. SSH and microarray analysis are powerful tools that can be used to achieve this objective. It is crucial to determine what genes are differentially expressed between the resistant and susceptible plants and which among the genes play an important role in plant defense. The identification and characterization of defense-related genes will lead to a better understanding of the molecular basis of wheat defense to *F. graminearum* infection, and will help in the development of transgenic FHB-resistant varieties.

CHAPTER II

DIFFERENTIALLY EXPRESSED GENES BETWEEN FHB RESISTANT AND SUSCEPTIBLE VARIETIES

Introduction

Wheat (*Triticum aestivum* L.) is a major cereal crop worldwide and used as a staple source of food by majority of world population. In humid and semi-humid wheat growing areas of the world, wheat head scab is the most destructive disease (Shroeder and Christensen, 1963). It is primarily caused by *Fusarium graminearum* and hence, is also referred as *Fusarium* head blight (FHB). Infection by this pathogen causes grain shriveling and consequently, there is a significant reduction in kernel weight. The infected kernels can be easily blown away with the chaff during threshing because of their lightweight. Indirect losses also come from infected seeds that germinate poorly, have seedling blight and poor stand (Bai and Shaner, 1994). Aside from reducing grain yield and quality, additional losses come from contamination of grains with mycotoxins produced by *F. graminearum* (Snijers, 1990). The toxins deoxynivalenol (DON) and zearelenone are harmful to animal and human health (Desjardins and Hohn, 1997). In North America, several epidemics in recent years resulted to a loss of more than $1 billion per year (McMullen et al. 1997). In China, Wang et al. (1992) estimated yield losses from 20% to 40% during severe epidemics. There is no effective chemical to control this disease.

The source of FHB resistance used by most breeding programs in the USA is Sumai 3 or its derivative, Ning 7840 (Chen et al. 2000). Although resistant to FHB, both have poor agronomic characters and transferring the resistance genes only by traditional breeding methods is difficult and time consuming. A novel approach was attempted to transform wheat with anti-fungal protein genes (Chen et al. 1999, Okubara et al. 2000). Chen et al. (1999) transformed wheat cv. Bobwhite with a rice *chi11* (chitinase) and *TLP*

(thaumatin-like protein), and obtained transgenic plants with delayed development of FHB symptoms. Okubara et al. (2000) incorporated six different anti-fungal genes into wheat. The highest level of gene expression was observed for *tlp-1* (isolated from wheat) while the anti-fungal protein genes isolated from lower species (*F. veneratum* and yeast) showed relatively low levels of transgene expression. There are only a limited number of anti-fungal genes available and most of them are not from wheat (Dahleen et al., 2001).

As a response to pathogen infection, plants express a wide array of genes and among these genes are the pathogenesis-related (PR) genes. A limited number of PR genes have been documented in wheat. Pritsch et al. (2000) studied the expression of six defense-related genes in wheat. Using two probes from wheat (peroxidase and β-glucanase) and four from barley (β-glucanase, chitinase and two TLPs), they reported that expression of the genes occurred as early as 6 hours after inoculation (hai) with *F. graminearum* and was the highest at 36 to 48 hai. Results also showed that transcripts of TLPs were detected earlier in the resistant cultivar Sumai 3 than in the susceptible cultivar Wheaton. Xing et al (2000) compared the expressed sequence tags (ESTs) profile of Sumai 3 and Wheaton and identified three ESTs that were only expressed in *F. graminearum*-inoculated Sumai 3. These ESTs were proposed as defense-related.

Li et al. (2001) reported the isolation of chitinases and β-1,3-glucanases from *F. graminearum*- infected wheat spikes using barley chitinase and β-1,3-glucanase as well as rice chitinase as probes. Expression analysis revealed that transcripts of these genes accumulated after infection by the pathogen during the first 24 hours, and a higher level of expression was observed in Sumai 3 (resistant) than in its susceptible mutant. Based on knowledge gathered to date, key genes for resistance to FHB have not been identified in

wheat. To genetically engineer FHB-resistant plants and make the resistance durable, the molecular mechanism of wheat-*F. graminearum* interaction must be well understood and key resistance pathways have to be identified. Genome-wide analysis of gene expression in response to *Fusarium* infection may help to understand genetic mechanisms of wheat resistance to FHB and identify key genes to control FHB resistance. It is crucial to determine what genes are induced during early infection and differentially expressed between the resistant and susceptible plants, and which among these genes may play an important role in plant defense. DNA microarrays allow the simultaneous analysis of thousands of genes in one experiment. It enables the detection and identification of genes that are differentially expressed in genotypes with contrasting responses to pathogen infection. Cluster analysis of microarray data may identify groups of genes that demonstrate the same pattern of activation or repression of major regulatory pathways. The identification and characterization of defense-related genes will lead to a better understanding of the molecular basis of wheat defense to *F. graminearum* infection, and will help in the development of transgenic FHB-resistant varieties. Resistance gene(s) can be engineered to over-express in transgenic wheat to enhance FHB resistance (Dahleen et al. 2001).

The specific objectives of this study are to construct PCR-based cDNA libraries of differentially expressed genes involved in FHB resistance and identify differentially expressed genes between Ning 7840 and Clark during *Fusarium* stress.

Materials and Methods

Plant materials

Near-isogenic lines (NILs) were not yet available at the start of the experiment. In lieu of NILs, bulked segregants from a population of $F_{9:12}$ recombinant inbred lines (RILs) derived from Ning 7840 x Clark were used to construct the SSH libraries. Ning 7840 is resistant to FHB, while Clark is susceptible to the disease (Figure 2.1). Five resistant RILs (RILs 13, 24, 86, 112 and 132) and five susceptible RILs (RILs 7, 15, 33, 52 and 101) were chosen for bulk construction based on their FHB evaluation from five greenhouse tests (Bai et al. 1999). Since the lines were selected based only on their response to FHB infection, it was assumed that other genes are randomly distributed among these selected lines and that the two bulks differ only in the genes involved in FHB resistance.

To generate infected wheat spike samples, wheat seedlings were vernalized in a germination tray for 6 weeks at 4 °C in a growth chamber, transplanted into 5 1/4" dura-pots (Hummert Int, Earth City, MO) and grown in a growth chamber at 20°C under 12 h of light and 15°C for 12 h under darkness.

Conidiospore production

Forty grams of mungbean seeds were boiled in 1 liter of water for 10 min. After boiling, the mixture was passed through two layers of cheesecloth to filter out the mungbean. The mungbean broth was aliquoted into flasks and autoclaved for 30 min. A small (ca. 0.5 x 1.0 cm) plug of *F. graminearum* agar was inoculated into a 100 ml mungbean medium and grown for four days at 28 °C with shaking (240 rpm). After the

growing period, the culture was passed through two layers of cheesecloth to remove the mycelium. Spores were counted using a haemacytometer and inoculum concentration was adjusted to 100 spores/µl. The inoculum was stored at 4 °C until use.

Inoculation of wheat spike and mRNA isolation

At anthesis, 10 µl of *F. graminearum* conidiospore suspension (100 spores/µl) was inoculated into the floral cavity between the lemma and palea of a central floret of a spike. Uninoculated plants and plants inoculated with mungbean broth medium alone (without pathogen) served as controls. Control plants were treated exactly the same way as the experimental plants. Sandwich bags sprayed with water were used to enclose the inoculated spikes until sampling or 72 hai, whichever came first. All inoculated plants were grown in a growth chamber at 25°C under 18 h of light and at 22°C for 6 h in darkness. Wheat head spikes were collected at 6, 36 and 72 h after inoculation (hai), immediately frozen in liquid N_2 and stored at -80 °C until use. Prior to sample collection, the inoculated floret was removed to exclude the possibility of extracting fungal RNA. High purity and quality mRNA was extracted from wheat spikes obtained from aforementioned treatments using the Message Maker Kit (Invitogen, Carlsbad, CA). For SSH library construction, RNA was extracted from tissues of bulked susceptible or resistant lines (0.5 g/line).

SSH library construction

Suppression subtractive hybridization (SSH) was done using the PCR-select cDNA subtraction kit from Clontech (Palo Alto, CA). Tissues collected at 6, 36 and 72 hai were used to generate three sets of forward and reverse SSH libraries, respectively.

Each forward library was constructed using the inoculated resistant RILs as the tester and the inoculated susceptible as the driver. In the reverse library, infected susceptible RILs served as the tester and infected resistant RILs as the driver.

Driver and tester preparation

First strand cDNA was synthesized by reverse transcription from 2 μg mRNA following the protocol from the PCR-select cDNA Subtraction Kit (Palo Alto, CA). Then second strand cDNA was synthesized and the ends of DNA fragments were blunted using T4 DNA polymerase. The dsDNA was digested by *Rsa I* restriction enzyme, purified by phenol extraction, and precipitated with ethanol. After resuspension in 5 μl of distilled water, the tester cDNA was ligated to two different adapters in two separate reactions. Ligation was done overnight at 16 °C with the aid of T_4 DNA ligase. Adapters were not ligated to the driver cDNA.

Subtractive hybridization

Two separate hybridization reactions started with mixing 20 ng adapter1-or adapter2-ligated tester cDNA with approximately 600 ng driver dsDNA (Appendix A). After ethanol precipitation and resuspension in the hybridization buffer, the cDNAs were denatured at 98 °C for 15 minutes and then incubated at 68 °C for 2 h for annealing to occur. The two reactions were added to 150 ng pre-denatured fresh driver cDNA in a single tube for second hybridization at 68 °C for 10 h. The hybridization mixture was then diluted with dilution buffer and heated at 72 °C for 7 min prior to storage at 20°C.

PCR amplification

Two PCRs were performed using the subtracted hybridization mixtures described above as templates. The first amplification was done using the following profile: 75 °C for 5 min, followed by 27 cycles of 94 °C for 30 sec, 66 °C for 30 sec and 72°C for 1.5 min. One µl of a ten-fold dilution of the product was used in the next amplification using nested PCR primers 1 and 2 from the SSH kit. The reaction was subjected to 10-12 rounds of denaturation at 94 °C for 30 sec, annealing at 68 °C for 30 sec and extension at 72 °C for 1.5 min. The amplification products was analyzed by gel electrophoresis and then inserted into a pT-Adv cloning vector (Clontech, Palo Alto, CA) or pGEM-T easy vector (Promega Corporation, Madison, WI).

Transformation

Transformation was done following the heat shock method described in the Clontech and Promega manuals. Two µl of the ligation reaction was mixed with 50 µl competent cells. Cells were incubated in an incubator at 37°C by shaking at 225 rpm for 1 hr after SOC medium was added. Cells were plated on LB/ampicillin/X-gal plates and incubated overnight at 37°C. White colonies were picked and grown in a liquid LB-ampilcillin medium for bacterial stock preparation and PCR amplification or plasmid isolation.

Gene expression analysis by microarrays

Fabrication of cDNA array slides with SSH clones

Inserts in cloning vector were amplified via PCR in 100 µl total reaction volume consisting of 4 µl fresh bacterial cells containing target PCR fragments, 1X PCR buffer,

1.5 mM $MgCl_2$, 0.24 mM dNTPs, 0.26 µM M13 forward primer, 0.26 µM M13 reverse primer, and 2 U of Taq polymerase. All PCR products were analyzed in 1% agarose (w/vol) gel in 1X TAE buffer. Only clones that produced bright and sharp PCR products were included in the microarray experiment (Figure 2.2). In addition, inserts smaller than 150 bp were also excluded. The PCR products (total of 2306) were precipitated with 3M NaOAC (pH at 5.2) and two volumes of ethanol. The resulting pellet was resuspended in 3X SSC at a final concentration of 100 ng/µl. The PCR products, actin gene (positive control) and vector with no insert (negative control) were arrayed on GAPS II coated slides (Corning, Corning, NY) using Array Spotter Generation III (Molecular Dynamics, Sunnyvale, CA) at Oklahoma University, Norman, or using OmniGrid100 Microarray Printer (GeneMachines, San Carlos, CA) at the Microarray Core Facility, Oklahoma State University. All samples were printed at least twice in a single slide. In addition, 2500 clones from 6 h and 48 h wheat root aluminum stress libraries were also printed in the same slide. Complimentary DNAs were immobilized in the glass slides by cross-linking at 300 mJoules using a Stratalinker (Stratagene, La Jolla, CA). Prior to hybridization, the array was rehydrated by placing it over a beaker with boiling water for a few seconds with the array side facing down. Once a thin layer of mist covered the slide surface, the slide was removed and dried briefly by placing the slide face up on a hot plate (30 sec) then baking at 80°C in a oven for 3 h. Slides were stored desiccated *in vacuo* at room temperature until use. After batch printing, a sacrificial slide was stained with syto-61 dye to visualize the quality of printing (Figure 2.3).

RNA extraction and cDNA synthesis

F. graminearum-inoculated spikes of Ning 7840 and Clark were sampled at 0, 3, 6, 12, 24, 36, 48 and 72 hai. Two wheat spikes per sample were pooled for RNA extraction. RNA was isolated using Trizol reagent (Invitogen, Carlsbad, CA). The 3DNAArray 50^{TM} kit from Genisphere (Genisphere Inc., Hatfield, PA) and SuperScript II enzyme (Invitrogen, Carlsbad, CA) was used for first-strand cDNA synthesis and hybridization. Two separate cDNA synthesis reactions were performed for each sample. Each reaction mix contained 40 µg total RNA, 400 U Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA), 1X first strand buffer, 0.01 mM DTT, 0.5 mM dNTPs and 3 µl of reverse transcription primer with a capture sequence at the 5'-end. The reaction mix was incubated at 42°C for 2 h. To stop the reaction and degrade the mRNA from the cDNA:mRNA hybrid, the reaction mix was treated with 7 µl of 0.5 M NaOH/50 mM EDTA and incubated at 65°C for 10 min. To neutralize the reaction, 10 µl of 1M Tris-HCl (pH at 7.5) was added. The cDNAs from the experimental and control samples were combined in a single tube. Sixteen µl of 10 mM Tris (pH at 8) and 1 mM EDTA was added to the reaction mix prior to clean-up using Amicon microcon-30 (Millipore Company, Billerica, MA).

Array hybridization

In the first hybridization, cDNAs were hybridized to the cDNA arrays, and in the second, 3DNA capture reagents #1 (Cy3) and #2 (Cy5) were hybridized to the 5'-end of the cDNAs to label them.

Slides were first washed with 0.1% SDS for 2 min, and then in water for another 2 min. After boiling for 3 min, slides were rinsed in 95% ethanol and then centrifuged to

dryness. A mixture consisting of 10 µl concentrated cDNA, 1X formamide-based hybridization buffer, and 2 µl of dT blocker was incubated at 78°C for 10 min and then at 48°C for 15 min. The reaction mix was added directly onto a pre-warmed glass slide (45°C) and a cover slip was carefully laid on top of the slide. The slide was incubated overnight at 45°C in a hybridization chamber (Corning, Corning, NY). After hybridization, the slide underwent three sequential 15-min washes (2X SSC plus 0.2% SDS; 2X SSC; 0.2X SSC) at room temperature, rinsed in 95% ethanol for 2 min, dried by centrifugation and stored in slide holder.

The second hybridization mix, consisting of 1X formamide-based hybridization buffer and 2.5 µl each of 3DNA capture reagent #1 and #2, was incubated at 78°C for first 10 min and then at 48°C for an additional 15 min. The mix was added to the microarray slide (from first hybridization). The slide was pre-warmed at 50°C for 12 min. before use. After a cover slip was placed on the surface of the slide, the slide was wrapped in aluminum foil and was placed in a hybridization chamber with a temperature setting of 45°C for 2 to 3 h. After hybridization, the slide underwent three sequential stringent washes (2X SSC plus 0.2% SDS; 2X SSC; 0.2X SSC) at room temperature for 15 min in each wash. The slide was dried by centrifugation and stored in a light-tight slide holder until scanned.

The experiment was repeated twice for each pair of biological samples at the various time points of the fungal stress. Dye labeling was reversed between two paired samples from two separate experiments to avoid bias in the microarray evaluation as a consequence of dye-related differences in fluorescence signal or hybridization.

Data collection and analysis

The signal intensity for each array was captured by scanning the slides at two wavelengths (532 nm for Cy3 and 635 for Cy5) using ScanArray Express (Perkin Elmer, Wellesley, MA) or GenePix 4000B microarray scanner (Axon Instruments, Union City, CA) at pixel size resolution of 10 microns. The laser setting was 100% and PMT setting varied with the intensities of the spots because saturation of pixels will reduce data accuracy.

Dyes Cy5 and Cy3 were scanned through two separate channels in a microarray scanner. The two channels were assigned the red and green colors, respectively, in the scanned images. The two images were combined and spot fluorescence was analyzed using GenePix Pro 5.0 software (Axon Instruments, Union City, CA). Feature indicators were placed on spots to distinguish features from the background. The background signal was subtracted from the fluorescence signal of the spot to obtain spot intensities. Spot intensities based on the median of background-subtracted signal were used because the median is less influenced by outliers compared to the mean. The composite image was visually inspected for spots with scratch and dust particles and were excluded from further analysis by flagging as "bad". Spots that did not have at least 55% of the feature pixels greater than one standard deviation above background in either red or green channel (spots with low intensity) were automatically flagged "bad". In addition, features that were not uniform were also excluded from downstream analyses.

Gene expression levels were quantified based on the ratio of signal intensities between the experimental and control samples. Intensity ratios were expressed in log base 2 ratios. Log2 ratios of "+1" and "+2" mean that the particular gene in the experimental

sample is up-regulated 2-fold and 4-fold, respectively relative to the control sample, while "-1" and "-2" mean that the gene is down-regulated 2-fold and 4-fold, respectively, relative to the control. Excel data generated from GenePix Pro 5.0 (in text tab-delimited format) were uploaded into GenePix Pro Auto-Processor (Weng and Ayoubi, 2004; http://darwin.biochem.okstate.edu/gpap). The baseline value of signal intensity was set to 200 and any sample with signal intensity less than 200 in both channels was filtered out. In addition outliers among replicates (spots with signal intensity greater than or less than the mean log2 ratio + 2SD) were also filtered out. Samples were normalized using Lowess scaled normalization method. The log2 ratio of replicated spots was averaged and differentially expressed genes were ranked from the highest to the lowest based on statistical significance (B statistics). Genes with a B value of at least 5 and differing in expression level by at least 1.5 times that of the control were considered as significantly differentially expressed. In addition, only those genes with significant log2 ratios averaged from at least four out of six data points were considered significant. In cases where there were only four data points available, a significant log2 ratio averaged over three data points were also considered significant. Moreover, the expression of a particular gene in both slides must show the same trend in expression pattern: either up-regulated in the two replicates or down-regulated in both. Genes with similar expression patterns were grouped together using K-means clustering with Genesis software (Sturn et al. 2002).

Gene sequencing and identification

Bacteria with cloned EST fragments from glycerol stocks were picked and re-grown in 96-well plates with Circle grow media (Bio 101, San Diego, CA). The plates were incubated for 20 h at 37°C with shaking at 200 rpm. Plasmid DNA was extracted using QiaPrep Turbo 96 BioRobot Kit (Qiagen, Valencia, CA) and DNA sequencing was performed using M13 universal primer. Reactions were run either in a 3700 ABI automated sequencer (DNA Sequencing Facility, Kansas State University) or in a Li-Cor 4200 DNA analyzer using DYEnamic Direct Cycle Sequencing Kit (Amersham Biosciences, Piscataway, NJ). Vector sequences were removed from the raw sequence and a BLASTX sequence homology search (Altschul et al. 1997) was performed. The highest similarity score generated by BLAST was considered the best match and was used as the putative identity of corresponding ESTs. Sequence similarity was considered significant if E-value was equal to or less than 0.001.

Results

SSH library and microarray analysis

A total of 2306 clones were isolated from 6, 36 and 72 hai SSH library pairs. The number of clones obtained from each library is summarized in Table 2.1. A total of 1047 clones were obtained from the 6 hai libraries whereas ca. 600 were obtained from each of the two libraries. DNA sequence analysis of differentially expressed ESTs revealed 5 redundant ESTs with 2-7 copies each (Table 2.2).

A portion of a slide hybridized with cDNAs from Ning and Clark sampled at 36 hai is shown in Figure 2.4. A total of 199 significantly differentially expressed ESTs were

identified between Ning 7840 and Clark (Appendix B). The number of differentially expressed ESTs from the libraries derived from FHB and aluminum stressed wheat materials were 117 and 82, respectively. The criteria used to determine the differentially expressed genes was set at a B value of at least 5 and difference in expression level of at least 1.5 fold between fungal-inoculated resistant and susceptible varieties. A B value of 5 means that the probability that a gene is differentially expressed in a fungal infected spike is 10^{-5} times higher than that if the gene has the same level of expression as in the control.

The highest number of up-regulated ESTs was observed at 3 hai (Figure 2.5). Results indicated that there were more differentially up-regulated ESTs than down-regulated ESTs during the first 24 hai with the pathogen, but more significantly down-regulated ESTs were observed at 36 hai and after. The distribution of down-regulated genes was bimodal. It initially peaked at 3 hai, dropped at 6 hai and increased thereafter. The greatest difference between the numbers of up- and down-regulated ESTs was at 6 hai with the number of up-regulated ESTs higher by 2.5-fold. In contrast, the number of down-regulated ESTs was higher by 2.2-fold compared to up-regulated ESTs at 72 hai. The least difference in the number of up- and down-regulated ESTs was at 36 hai.

Figure 2.6 shows the putative functional classification of all 199 differentially expressed ESTs based on sequence homology analysis using the BLAST program. Only about half of the differentially expressed ESTs have homology with known accessions in GenBank. Defense-related ESTs are only about 9% and 24% are involved in protein destination, photosynthesis, transport facilitation and transcription regulation. Genes that are involved in protein folding and stabilization, protein targeting, sorting and

translocation, protein modification and proteolysis were classified in protein destination. About 19% of ESTs screened are either with unknown function or hypothetical proteins.

Expression profiles of differentially expressed ESTs

Differentially expressed ESTs were grouped into clusters using the Genesis software (Sturn et al, 2002). Twelve clusters (Figures 2.7-2.11) were generated according to similarities in expression patterns using K-means method. Based on their expression patterns, these 12 clusters are further regrouped into six major groups: (I) EST up-regulated in most time points investigated, (II) EST up-regulated at few time points investigated, (III) ESTs up-regulated mainly at early time points and down-regulated at later time points of fungal stress; (IV) ESTs down-regulated during all time points studied; (V) ESTs down-regulated mainly at early time points of fungal stress; and (VI) ESTs down-regulated mainly at late time points of stress.

Clusters 3, 10 and 4 belong to group I. All ESTs in these two clusters showed up-regulation in most time points of fungal stress except a few ESTs in cluster 4 which showed down-regulation in very late time points of fungal stress. Cluster 3 contains five ESTs and they are all strongly up-regulated at eight time points of fungal stress. These ESTs may play an important role in enhancing FHB resistance in Ning 7840, although their function is unknown.

Cluster 10 is the biggest cluster with 50 up-regulated ESTs. Those ESTs also showed up-regulation in most time points, but with lower expression level in comparison with the ESTs in cluster 3. Some of the ESTs have similarity with genes involved in sucrose (SPP1) or starch biosynthesis (G-6-P adenylyltransferase), production of fatty alcohols and aldehydes (fatty acid CoA reductase), protein bond formation (protein

disulfide isomerase), and protein turnover (serine carboxypeptidase). Most of the ESTs in this cluster have no significant similarity with known genes.

Cluster 4 has 12 ESTs that are up-regulated at most time points with few of the ESTs weakly down-regulated at 36 or 72 hai. Peak of up-regulated expression is at 12 hai. This group includes ESTs for glyceraldehyde-3-phosphate dehydrogenase and ribulose bisphosphate carboxylase (Rubisco), ß-glucosidase, B2 protein, putative kinesin and several unknown proteins.

Group II includes clusters 7 and 9 in which most ESTs showed up-regulation in few time points of fungal stress. ESTs in cluster 7 were up-regulated at 3 hai and had mixed expression pattern or no differential expression at other time points of fungal stress. This group of ESTs may be involved in cell wall modification and synthesis of signaling compounds which include ESTs for lignin biosynthesis, amino acid biosynthesis and fatty acid oxidation. ESTs in cluster 9 were mainly up-regulated at 6 hai and 12 hai with a few down regulated ESTs at 0 to 3 hai. Some of ESTs in this cluster are similar to carbonic anhydrase, Rubisco, zinc finger and wheat pollen allergen homolog. Other ESTs in these two clusters had no significant similarity with genes in public databases.

Group III consists of Clusters 1, 2 and 11. ESTs in this group are predominantly defense-related genes, which were up-regulated initially and then down-regulated at later time points of fungal stress.

Cluster 1 is made up of 38 ESTs. The baseline expression levels of these ESTs (0 h) show that most of them were already up-regulated in Ning 7840 relative to Clark before inoculation of the pathogen. These ESTs were still up-regulated at 3 hai but

expression was lower compared to 0 h (uninoculated). Expression of these ESTs continued to decrease and most were significantly down-regulated at 36-72 hai. ESTs in this cluster have significant similarity with known defense-related genes such as P450s, PR proteins (PR-1, chitinase, TLP) and alternative oxidase. Two ESTs were putatively identified as MRP-like ABC transporter and high affinity potassium transporter. Twenty ESTs in this cluster do not have significant homology to other genes in GenBank.

There were seven and five ESTs in clusters 2 and 11 respectively. Only two ESTs in the cluster 2 have homology with glutamine-dependent asparagine synthetase and pathogenesis-related protein, while others either had no homology to known genes in the existing GenBank database or sequences were not determined. In cluster 11, three ESTs had similarity to cadinene synthase and proteinase inhibitors. Most of these ESTs were not differentially expressed at 0 h.

Group IV contains 15 ESTs from cluster 5 and seven ESTs from cluster 12. ESTs from cluster 5 were all down-regulated in most time points of fungal stress. Some were putatively identified as proline oxidase, MADS-domain transcription factor and putative NPR1 regulatory protein. Seven ESTs in this cluster had no sequence homology with genes in public databases while two ESTs share significant sequence similarity with unknown or hypothetical proteins. ESTs from cluster 12 were mainly down-regulated from 6 to 36 hai and differential expression was not obvious for other time points. Three ESTs in this cluster were identified as putative ß-amylases and a putative transporter. Others had no significant similarity with genes in GenBank.

Cluster 6 belongs to group V. This group of ESTs showed down-regulation during early fungal stress, (0 to 6 hai). At later time points, some were still down-regulated but

others showed up-regulation or no differential expression. Among 27 ESTs, a putative Rieske Fe-S precursor, chlorophyll A-B binding protein, Rubisco activase and small subunit, copper chaperone and MADS box proteins were identified**.** The majority of these ESTs are involved in photosynthesis.

Other clusters

The last group is cluster 8. It is composed of five ESTs that are significantly down-regulated at 72 hai although mixed expression patterns were observed for previous time-points. EST G5-264 had similarity with an auxin-repressed protein-like protein with unknown function. The other EST, G8-157 is similar to a wheat β-glucanase gene. One EST is similar to a protein with unknown function while the two others have no sequence similarity with known sequences in public databases.

Discussion

SSH library construction and data analysis

This study was designed to identify fungal-induced or repressed genes in the resistant and susceptible cultivars in response to *F. graminearum* stress. To achieve this objective, we constructed SSH libraries using fungal inoculated resistant and susceptible lines sampled at 6, 36 and 72 hai. Several studies have shown that the *Fusarium* macroconidia germinates after 6 hai, directly enters the top part of the ovary, inner lemma and inner palea 36 hai and spreads after 48 hai (Pritsch et al. 2000 & 2001; Kang and Buchenauer, 2000). These time points reflect important fungal activities and were chosen.

SSH was the technique of choice because it offers several advantages over other methods of isolating differentially expressed genes such as differential display or RDA.

SSH is a PCR-based method that involves a normalization step to balance the abundance of cDNAs in the target population and a subtraction step to remove cDNAs that are common between the target and tester populations (Diatchenko et al. 1996). These steps will reduce the number of redundant clones in a SSH library as well as the number of clones to screen because common cDNAs between the two populations are subtracted out. Another advantage of SSH is that it can enrich rare cDNAs (several molecules per cell) by more than 1000-fold, thereby enabling the isolation or rare transcripts that may not be easily obtained from a regular cDNA library. In the current study, only about 800 clones were isolated for each set of libraries, which tremendously reduced the number of clones to be screened by microarray analysis. In addition, among differentially expressed sequences, most are single copy, and only several ESTs were multi-copy. These results indicated that SSH is very effective in removing redundant cDNAs and reducing the number of non-target clones.

However, this method still has some drawbacks. One disadvantage is that it may generate small cDNA fragments due to digestion with *RsaI*. It is difficult to design good primers based on a short stretch of DNA sequence and consequently, cloning the full-length cDNA may be more difficult. In our study, although fragments as small as 100 bp were observed, the average fragment size was 370 bp. The length of most cDNA fragments ranged from 250 bp to 500 bp, which are appropriate for PCR amplification. Short cDNA fragments (less than 150 bp) were excluded from microarray analysis in this study. In addition, Diatchenko et al. (1996) found that different fragments of the same cDNA may vary considerably in terms of hybridization or amplification characteristics Thus, some fragments from a differentially expressed cDNA can be eliminated and other

fragments from the same cDNA can be enriched and isolated during the SSH procedure. In our experiment, only several redundant clones that shared identical DNA sequences were identified, and they exhibited the same expression pattern (Table 2.2). In such cases, only one representative clone was reported and the other duplicates were excluded from further data analysis.

Another drawback of SSH is that it produces fragments primarily from the 3'-end of the cDNA (Guilleroux and Osbourn, 2004). The C-terminal of end of a protein is not highly conserved and this may lead to failure in identifying a similar sequence in the GenBank database (Gracey and Cossins, 2003). This may be one of the reasons why BLAST-X did not find y significant homology to accessions in GenBank for many clones. Another reason could be that many genes in wheat are not yet discovered and therefore are not yet available in the GenBank.

The SSH libraries in this study were made from *F. graminearum*-inoculated resistant and susceptible RILs. Theoretically, any gene that was commonly induced as a response to fungal stress will be subtracted out, which include genes with abundant copies. With the removal of common sequences between FHB resistant and susceptible lines, SSH significantly reduced redundancy in the cDNA libraries and therefore, tremendously reduced the number of clones to be screened.

The number of clones obtained from the 6 hai libraries was greater than that from the 36 and 72 hai libraries (Table 2.1). The vector used in the former was pGEM-T Easy whereas pTAdv vector was used to construct the libraries of later time points. This may account for the difference in the number of clones obtained. In this study, only a very small subset of ESTs (2306) were derived from three SSH libraries in comparison with a

library of entire wheat genes in wheat genomes, which make it feasible for us to focus on target ESTs with limited resources.

Using an array printed with clones from these FHB SSH libraries and 2500 clones from wheat aluminum-stressed libraries, 199 transcripts were significantly differentially expressed between Ning 7840 and Clark, respectively. B-statistics was used to determine if an EST was significantly differentially expressed. This test is considered better than t-statistics because it takes into account the standard deviation, number of replicates and sample variance when averaging log2 ratios (Weng and Ayoubi, 2004). Classically, a gene was considered as significantly differentially expressed only if the difference in gene expression between experimental and control samples were at least 2-fold. In this study, we found that ESTs with only 1.5-fold difference is gene expression to be significantly differentially expressed based on B-statistics.

Out of the 199 differentially expressed ESTs identified in this study, 5 redundant clones were identified. The number of redundant clones was based only on a small subset of clones. If the entire collection of clones were sequenced, more redundant clones will probably be identified.

The number of differentially expressed ESTs from the libraries derived from FHB and aluminum stressed wheat materials were 117 and 82, respectively. The number from the aluminum library is surprisingly high and this implies that many genes identified in this study are probably common genes that can be induced by biotic and abiotic stresses. It is obvious that only about 5% of the ESTs from SSH libraries were differentially expressed even though the array was spotted with cDNAs from subtraction libraries. Obviously, most of the ESTs were not significantly differentially expressed. The results

indicate that the common sequences between the resistant and susceptible lines were not subtracted out as expected during the subtraction process. Therefore SSH is still not perfect although it is very efficient in removing redundant ESTs. However, this method greatly reduced the number of clones to be screened into a more manageable size which significantly reduced screening work and cost. The efficiency of this method in isolating differentially expressed genes will be improved significantly if it is combined with microarray analysis. Further analysis with microarray or differential expression techniques will provide a powerful tool to identify differentially expressed genes.

Defense-related ESTs

The twelve clusters generated from microarray analysis demonstrate various patterns of gene expression in Ning 7840 and Clark. These patterns reflect how Ning 7840 and Clark respond to *F. graminearum* stress. Based on these patterns, differentially expressed ESTs were divided into 6 groups.

Group II consisting of ESTs from clusters 1, 2 and 11 contains putative genes for plant defense. In this study, chitinase (PR-3) and TLPs (PR-5) were up-regulated in Ning 7840 at 0-3 hai. Chitinase (PR-3) degrades chitin in fungal cell walls (Collinge et al. 1993) whereas TLP (PR-5) is hypothesized to permeabilize fungal cell walls (Liu et al. 1994). In addition, digestion of chitin in fungal cell walls releases oligomeric products that can signal the plant to activate additional defense responses (Li et al. 2001). In spray inoculated wheat spikes, Pritsch et al (2000) reported that accumulation of PR-5 transcripts occurred at 6 hai in Sumai 3 (resistant) and 18 hours later in Wheaton (susceptible). In addition, more PR-5 transcripts were observed in Sumai 3 than in Wheaton 12 to 24 hai with the pathogen. Accumulation of PR-3 and PR-5 proteins were

observed in 6 to 12 hai and continued to accumulate 36 to 48 hai. Gradual accumulation of PR-3 transcripts was observed from 12 to 48 hai and was roughly the same for both resistant and susceptible cultivars. In contrast, our study shows that both PR-proteins were up-regulated at 0-3 hai. The difference in PR-3 and PR-5 gene expression in the study conducted by Pritsch et al. (2000) and our study could be due to the difference in inoculation method. Single floret inoculation delivers the pathogen directly into the floret cavity and will only have one initial infection site whereas spray inoculation could produce multiple infection sites.

Also in cluster 1 are ESTs that share some homology with (putative) P450 genes that belong to the CYP71C subfamily. These genes participate in the biosynthesis of 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), a major anti-fungal compound found in cereals (Frey et al. 1997). DIMBOA inhibits proteases and oxidative enzymes in fungi, bacteria and insects (Niemeyer, 1988).

The baseline expression level of the PR genes and P450s show that they were already up-regulated in Ning 7840 relative to Clark before *F. graminearum* inoculation. The ESTs were still up-regulated at 3 hai but transcript level was lower compared to endogenous level at 0 h. Expression of these ESTs continued to decrease and most were significantly down-regulated at 36-72 hai. The expression profile of these general defense genes indicates that they are probably not important to FHB resistance in Ning 7840. However, down-regulation means that gene expression in Clark is higher than in Ning 7840 and it is at 36 hai when these defense-related genes are up-regulated in the susceptible genotype. This implies that expression of these PR genes and P450s are part of the susceptibility response of Clark.

The up-regulation of genes with homology to PR proteins, proteinase-inhibitor related protein and cytochrome P450 homolog was observed in barley leaves in response to water stress (Ozturk et al.2002). According to Pritsch et al (2000), fungal spread from spikelet to spikelet probably occurs after 48 hai. As the pathogen spreads, the rachis gets clogged and nutrient and water supply to spikelets above the infected floret is limited or blocked. This suggests that fungal spread in the susceptible cultivar Clark may induce the expression of water stress genes. This indicates that expression of fungal-induced genes may also be triggered by water stress. Moreover, the induction of these ESTs in Clark occurred earlier than 48 hai and this implies that the response of Clark to water stress was probably superimposed with the activation of defense response in Clark.

Alternative oxidase and a PDR-like ABC transporter were also up-regulated at 0-3 hai in current study. The former is an enzyme that reduces mitochondrial reactive oxygen species (ROS) formation (Maxwell et al., 1999). On the other hand, a report on tobacco (Sasabe et al., 2002) showed that the expression of a NtPDR1 gene was induced by an elicitor. This suggests an involvement of some PDR genes in early plant defense.

Clones G1-159 and C150 are putative proteinase inhibitors. Studies conducted by Dunaevskii et al. (1997) showed that trypsin/chymotrypsin (a proteinase inhibitor) inhibits germination and growth of *Alternaria alternata* in buckwheat. Studies by Cordero et al. (1994) demonstrated the up-regulation of a proteinase inhibitor gene after wounding and fungal infection. Moreover, Richards et al. (1998) reported that high levels of aluminum induced proteinase inhibitors. This enzyme also disrupts the protein metabolism of insects that may attack after a plant is wounded (Hammond-Kosack and Jones, 2000). Clone 72R2 has homology with cadinene synthase from *Gossypium*

arboreum (tree cotton) and this enzyme is involved in the synthesis of gossypol, a secondary metabolite that inhibits germination of *V. dahliae* conidia (Banchini et al. 1999). Moreover, cadinene synthase was induced in cotton suspension cells 6 h after treatment with a purified fungal elicitor (Chen et al. 1996). Similarly, the cadinene synthase in this study was up-regulated at 3-6 hai. Proteinase inhibitors and cadinene synthase have been shown to inhibit germination and growth of pathogens and were up-regulated at 3 hai presumably to prevent germination of *F. graminearum.*

Genes that belong to the same cluster may have similar functions and may be co-regulated. The identities of the other 27 ESTs in this group did not produce any significant BLASTX hits but may also play a role in plant defense.

Differences in superoxide dismutase (SOD), catalase, phenylalanine ammonia lyase (PAL) and ascorbic acid oxidase activities between resistant and susceptible varieties have been reported by Chen et al. (1997) and Lu et al. (2001). SOD, catalase and ascorbic acid oxidase are involved in scavenging of reactive oxygen species. These enzymes are associated with senescence/cell death and will probably be expressed after the spread of *F. graminearum* and when the spikes are blighted. This is probably the reason why these ESTs were not differentially expressed in this study. On the other hand, the expression of PAL was not strong enough to meet the cut-off B value of at least 5.

ESTs involved in cell wall modification and synthesis of signaling compounds

At 3 hai, putative caffeic acid O-methyltransferase (COMT) and lipoxygenase (LOX) were up-regulated in Ning 7840 (cluster 7) in this study. COMT is a key lignin biosynthetic enzyme. Corn plants transformed with anti-sense COMT had reduced COMT activity and corn leaves had low lignin content compared to untransformed plants

(Piquemal et al. 2002). The possible role of lignin in FHB resistance is supported by the observation that the increase in lignin content of cell walls of *F. culmorum*-infected resistant plants was much greater compared to FHB susceptible plants (Kruger et al. 2002). Lignification of cell walls provides a stronger barrier against fungal penetration. One EST had similarity to LOX, a gene involved in jasmonic acid (JA)-induced lipid peroxidation in barley leaves (Bachmann et al. 2002). In addition, LOX generates secondary signal molecules such as peroxides that trigger additional defense response in plants. Up-regulation of COMT and LOX imply that Ning 7840 undergoes cell wall modification to prevent/inhibit fungal penetration.

Transcripts with homology to enoyl CoA hydratase/ isomerase were also up-regulated at 3 hai. Enoyl CoA hydratase/ isomerase is an enzyme involved in fatty acid oxidation. LOX and fatty acid oxidation enzymes are involved in the synthesis of JA, the signaling compound involved in most necrotrophic fungi-plant interactions (Thomma et al. 1998 & 1999; Staswick et al. 1998). These ESTs were not strongly up-regulated, but over expression may not be necessary for genes involved in signal transduction. Also up-regulated at 3 hai was a putative methionine synthase. Methionine is the only precursor for ethylene in plants. JA and ethylene are hormones that activate proteinase inhibitors and certain PR proteins (Buchanan et al. 2000). In addition, the transcript level of an EST with homology to a putative NPR1 (clone 36R359) was always significantly lower in Ning 7840 compared to Clark. NPR1 has been associated with salicylic acid- (SA) mediated responses (Despres et al. 2003). However, NPR1-independent SA signaling has also been reported by Clarke et al. (2000).

Several studies have shown that resistance to necrotrophs involves the JA/ethylene signaling pathway. Although there is no direct evidence that synthesis of JA and ethylene occurred, up-regulation of lipoxygenase, enoyl CoA hydratase/ isomerase and methionine synthase suggests that these hormones may be produced in Ning 7840 as a response to *F. graminearum* stress and that signal transduction is an early defense response.

Photosynthesis-related ESTs

ESTs having similarity to photosynthesis-related genes such as chlorophyll A-B binding protein, Rubisco activase and small subunit, and Rieske Fe-S precursor were down-regulated at 0-3 hai (cluster 6) but Rubisco and carbonic anhydrase were up-regulated from 6 –36 hai (cluster 4 and 9). Fungal induced down-regulation of these genes was probably necessary to re-allocate carbon flux toward defenses. Studies by Somssich and Halbrock (1998) showed the inverse relationship between expression of photosynthesis-related and defense-related genes. This is consistent with our findings that ESTs putatively involved in plant defense are up-regulated during the first 3 h after *F. graminearum* stress.

Other differentially expressed ESTs

A transcript with homology to ß-glucosidase was up-regulated at 0-6 hai. This enzyme hydrolyzes ß-glucosides resulting in the production of toxic aglycones and derivatives such isothiocyanates, nitriles, terpenoid alkaloids, saponins and hydrogen cyanide (Poulton, 1990). The enzyme and its substrate are normally located on different subcellular compartments but disruption of plant tissues by pest enables them to come in

contact with each other leading to synthesis of chemicals that inhibit the entry, growth and spread of pathogens.

Several ESTs that had similarity to genes involved in sucrose or starch biosynthesis, production of fatty alcohols and aldehydes, bond formation between proteins, and protein turnover were generally up-regulated for most of the time points (Cluster 10). These observations imply that the cellular processes involved in plant maintenance and development are more active in the resistant plant relative to the susceptible plant and this probably helps enable the fungal inoculated resistant plant to continue its normal growth and development.

Interestingly, a group of 5 ESTs (cluster 3) was found to be always strongly up-regulated in the resistant variety. All of them were similar to genes with unknown function. These ESTs may play an important role in FHB defense because they are always expressed at higher levels in the resistant variety compared to the susceptible variety. In addition, the down-regulated EST in the resistance response in cluster 5 may correspond to genes that are necessary for fungal spread. Further investigation of these ESTs may elucidate their role in FHB.

There were more differentially up-regulated genes than down-regulated ESTs during the first 24 hai after inoculation with the pathogen, but more significantly down-regulated ESTs were observed from treatments of 36 hai and onwards. The highest number of up-regulated ESTs was observed at 3 hai. In addition, the number of down-regulated genes showed a bimodal distribution that initially peaked at 3 hai. These observations suggest that 3 hai is an important time point in Ning 7840 defense response.

The identification of a large number of differentially expressed ESTs suggests that plant defense in response to FHB infection is complex and involves a regulatory network of genes involved in transcription, metabolism, energy generation, protein modification, cell rescue/defense as well as genes of still unknown function. Although a large percentage of these ESTs produced no hit in public databases or were similar to hypothetical or unknown proteins, similarities in gene expression pattern with known genes may provide possible insight on the function for novel or unknown proteins. Resistance to most necrotrophic fungi does not follow gene for gene resistance. One major QTL in 3BS chromosome arm plus a few other minor genes located in other chromosomes governs FHB resistance in Ning 7840. This study identified ESTs that are differentially expressed as a response to FHB infection. Although ESTs that had similarity to known genes were identified, the identity or function of the majority is still unknown. Physical and molecular mapping of these ESTs may provide important information on location of these genes and relationship between these genes and the 3BS major QTL. This may help to further understand the molecular basis of FHB resistance and lay a solid ground for cloning of FHB resistance genes.

Table 2.1. Total number of clones obtained from each library.

Time Point	Forward Library	Reverse Library
6h	625	424
36h	331	328
72h	315	283

Table 2.2. List of redundant differentially expressed ESTs

BLAST Hit	No. of Clones
ref|NP_914894.1| OSJNBa0052O12.12 [O. sativa]	7
ref|NP_914903.1| putative high-affinity potassium transporter [O. sativa]	5
dbj|BAD07637.1| putative cytochrome P450 [O. sativa]	3
ref|NP_922401.1| putative gibberellin oxidase	3
gb|AAO00709.1| putative gibberellin 20-oxidase [O. sativa]	2

Figure 2.1. Ning 7840 (left) and Clark (right) infected with *F. graminearum.* Plants were inoculated with *F. graminearum* conidiospores using the single floret inoculation method. Infection in Ning 7840 was limited to the inoculated floret (arrow), while infection in Clark spread over entire spike.

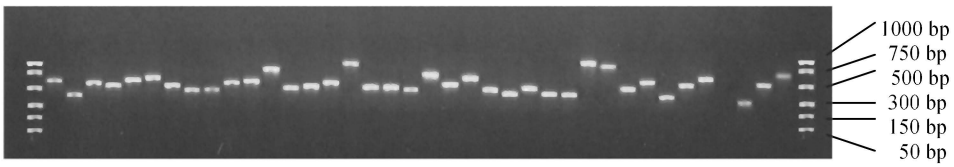


Figure 2.2. PCR products of cDNAs from the 36h SSH library. PCR amplification showed that the clones contained inserts with varied sizes. Average size was about 370 bp.

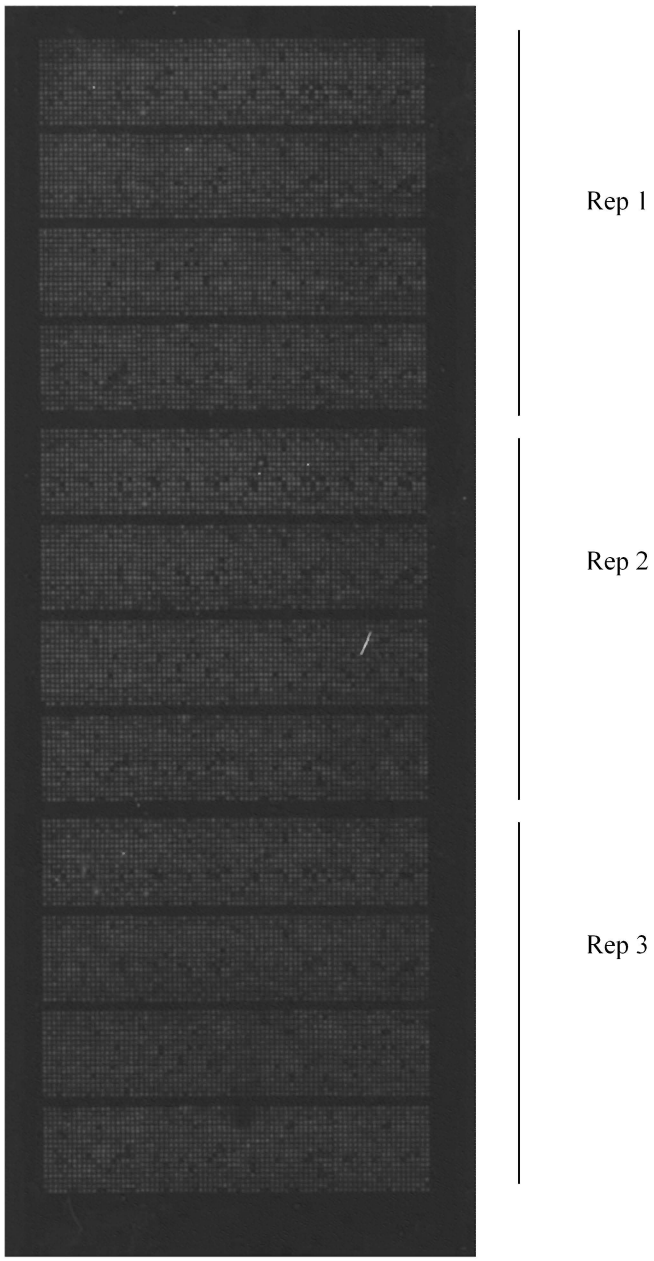


Figure 2.3. A syto-61 stained slide from a batch of slides printed using the OmniGrid II Arrayer (Gene Machines) at the OSU Microarray Core Facility. The red spots in this figure correspond to the cDNAs printed on the arrays. Each cDNA was printed in triplicate. Empty spots show the location of the negative controls (water or 3X SSC).

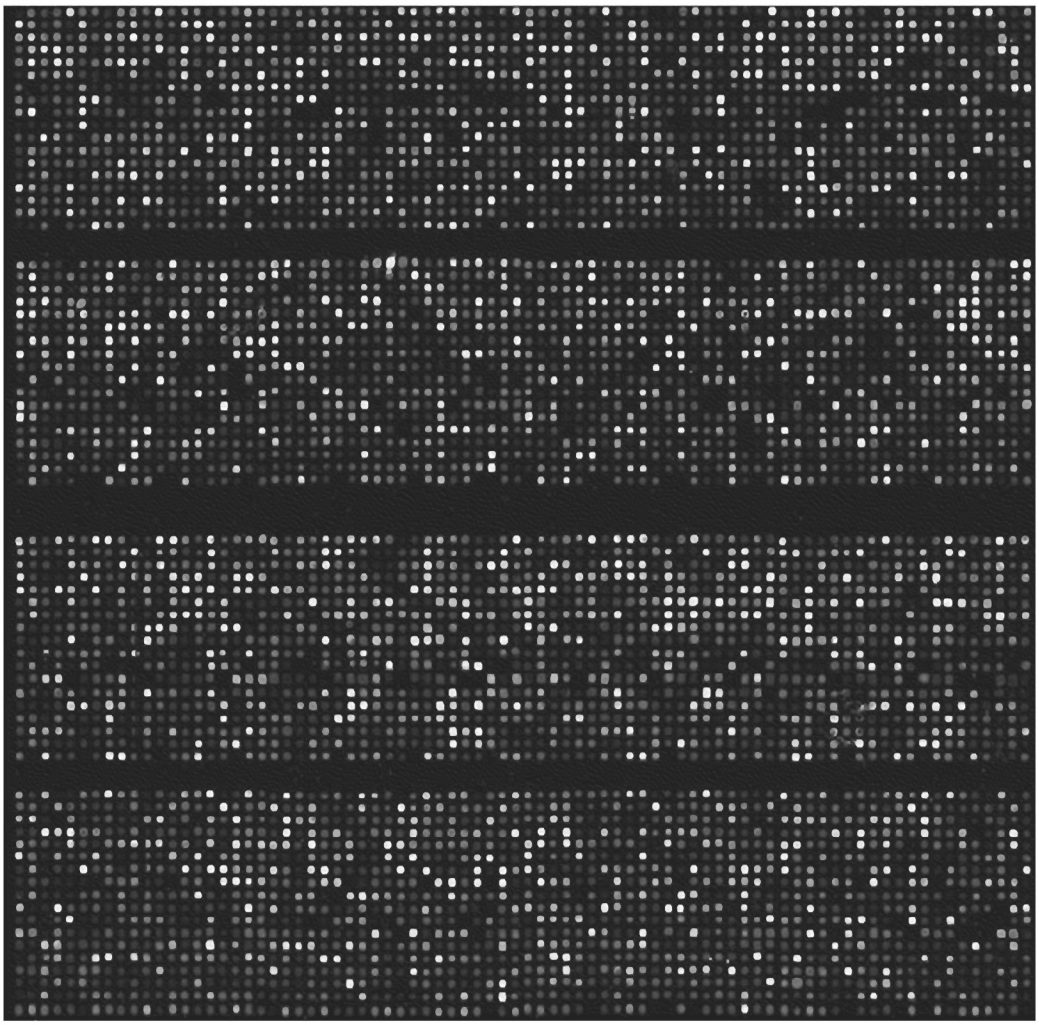


Figure 2.4. A pseudocolor image of a portion of a slide hybridized with cDNAs from Ning and Clark sampled at 36 hai with the *F. graminearum*. Slide-bound cDNA were hybridized with cDNAs from wheat spikes sampled at 36 hai with the pathogen. Complementary DNAs from Ning 7840 and Clark were labeled with cy5 (red dye) and cy3 (green dye), respectively. Up-regulated ESTs in the resistant variety are shown as red spots, down-regulated ESTs as green, and yellow spots correspond to non-differentially expressed ESTs between the resistant and susceptible varieties.

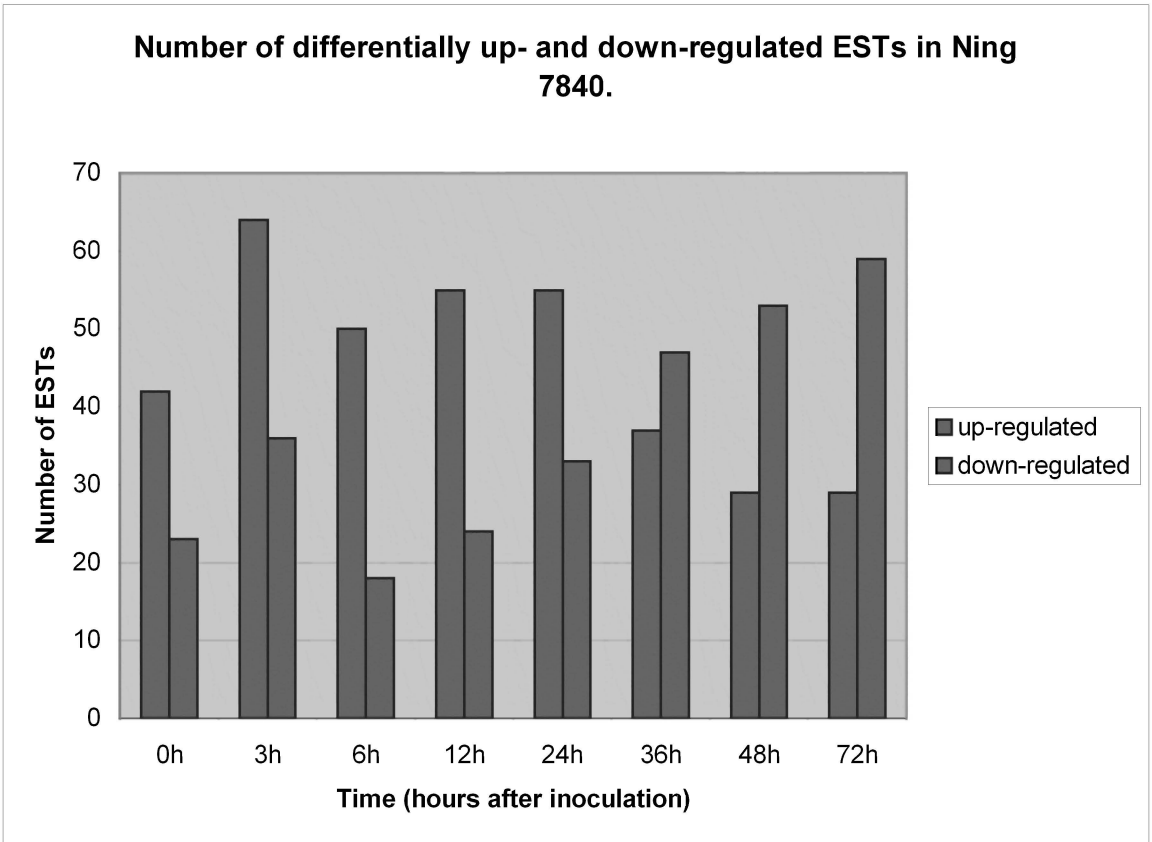


Figure 2.5. Number of differentially up- and down-regulated ESTs in Ning 7840. Red and green bars represent up-regulated and down-regulated ESTs, respectively. There were more differentially up-regulated ESTs than down-regulated ESTs during the first 24 hai with the pathogen, but more significantly down-regulated ESTs were observed from treatments of 36 hai and onwards.

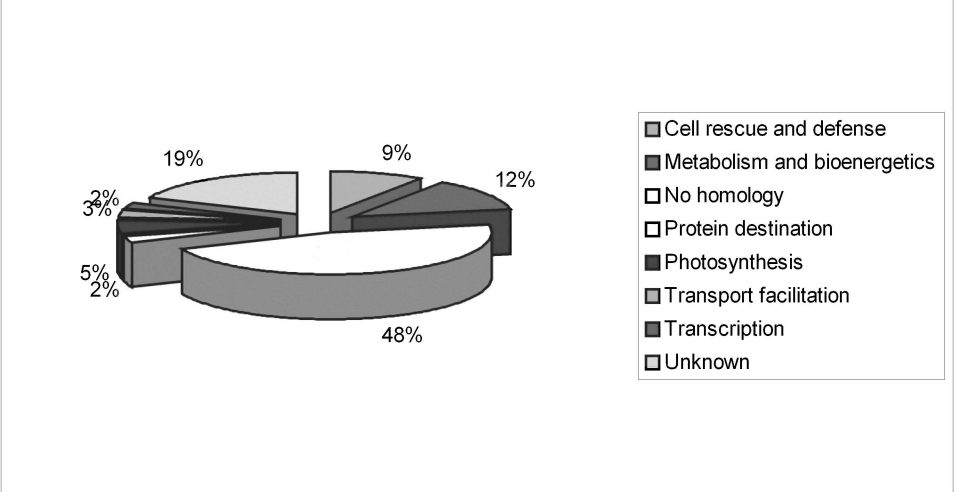


Figure 2.6. Functional classification of differentially expressed ESTs between Ning 7840 and Clark. Differentially expressed ESTs were sequenced and then compared to proteins in public databases by BLAST. The highest similarity score generated by BLAST was considered the best match and was used as the putative identity of corresponding ESTs.

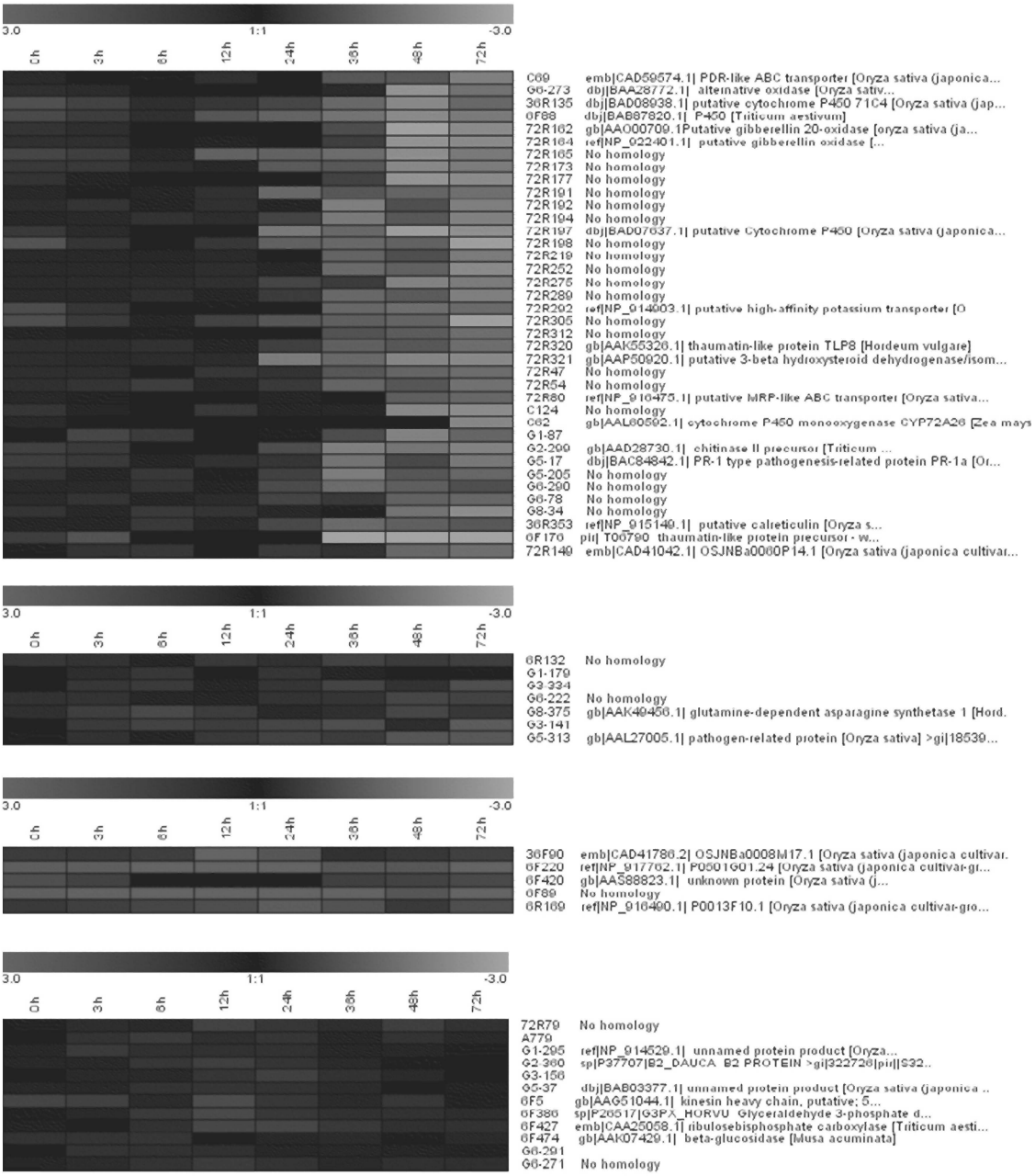


Figure 2.7. Clusters 1 to 4 of the differentially expressed ESTs between Ning 7840 and Clark. Each row represents one EST and each column corresponds to a particular time point. The scale of expression ranges (log2) from 3.0 (bright red) to –3.0 (bright green). Red boxes denote ESTs with expression levels significantly higher in the resistant variety relative to the susceptible variety. Green boxes mean that that particular EST is down-regulated in Ning 7840 (relative to Clark) at that particular time point. The highest

similarity score generated by BLAST was considered the best match and was used as the putative identity of corresponding ESTs.

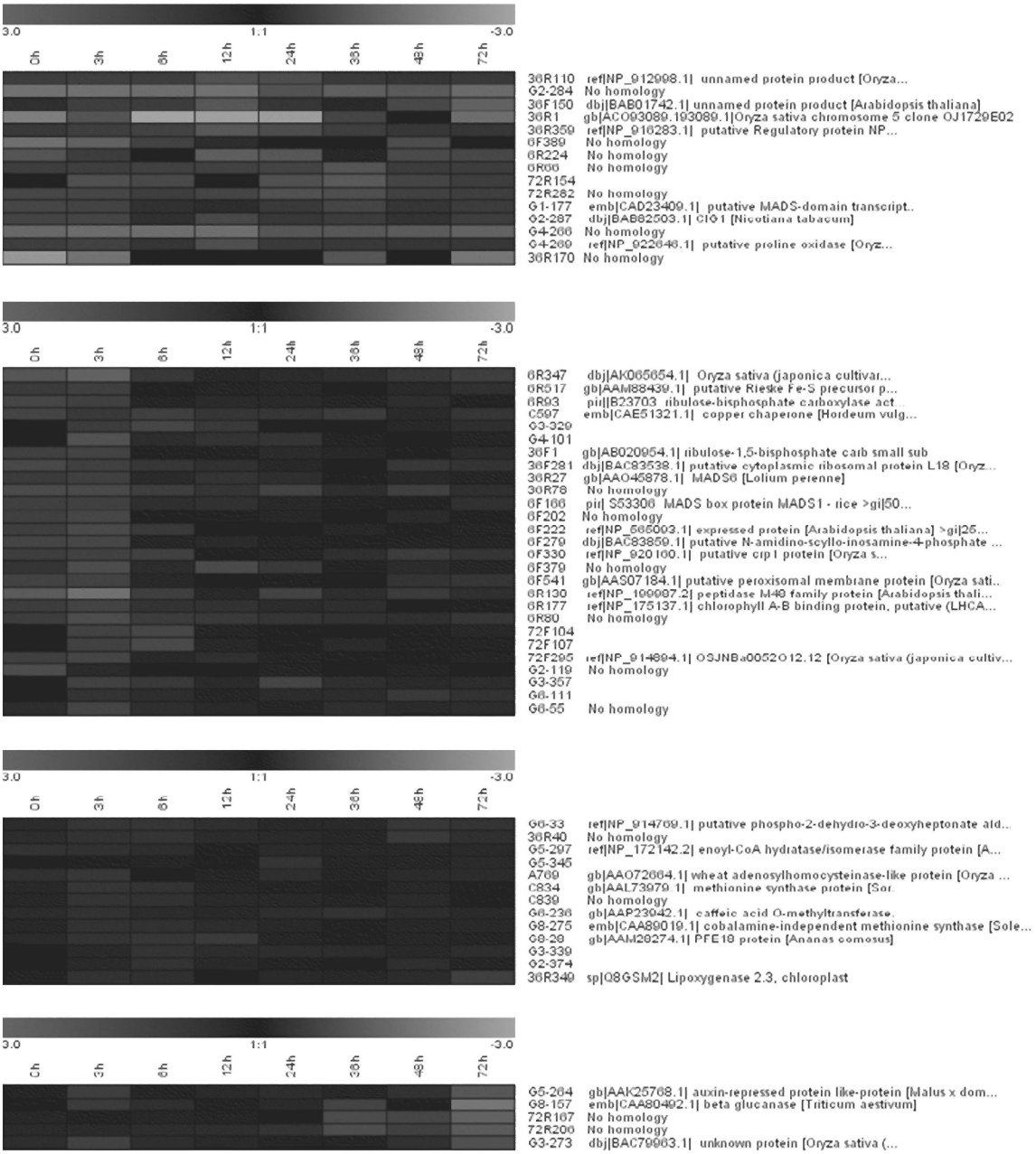


Figure 2.8. Clusters 5 to 8 of the differentially expressed ESTs between Ning 7840 and Clark. Each row represents one EST and each column corresponds to a particular time point. The scale of expression ranges (log2) from 3.0 (bright red) to –3.0 (bright green). Red boxes denote ESTs with expression levels significantly higher in the resistant variety relative to the susceptible variety. Green boxes mean that that particular EST is down-regulated in Ning 7840 (relative to Clark) at that particular time point. The highest

similarity score generated by BLAST was considered the best match and was used as the putative identity of corresponding ESTs.

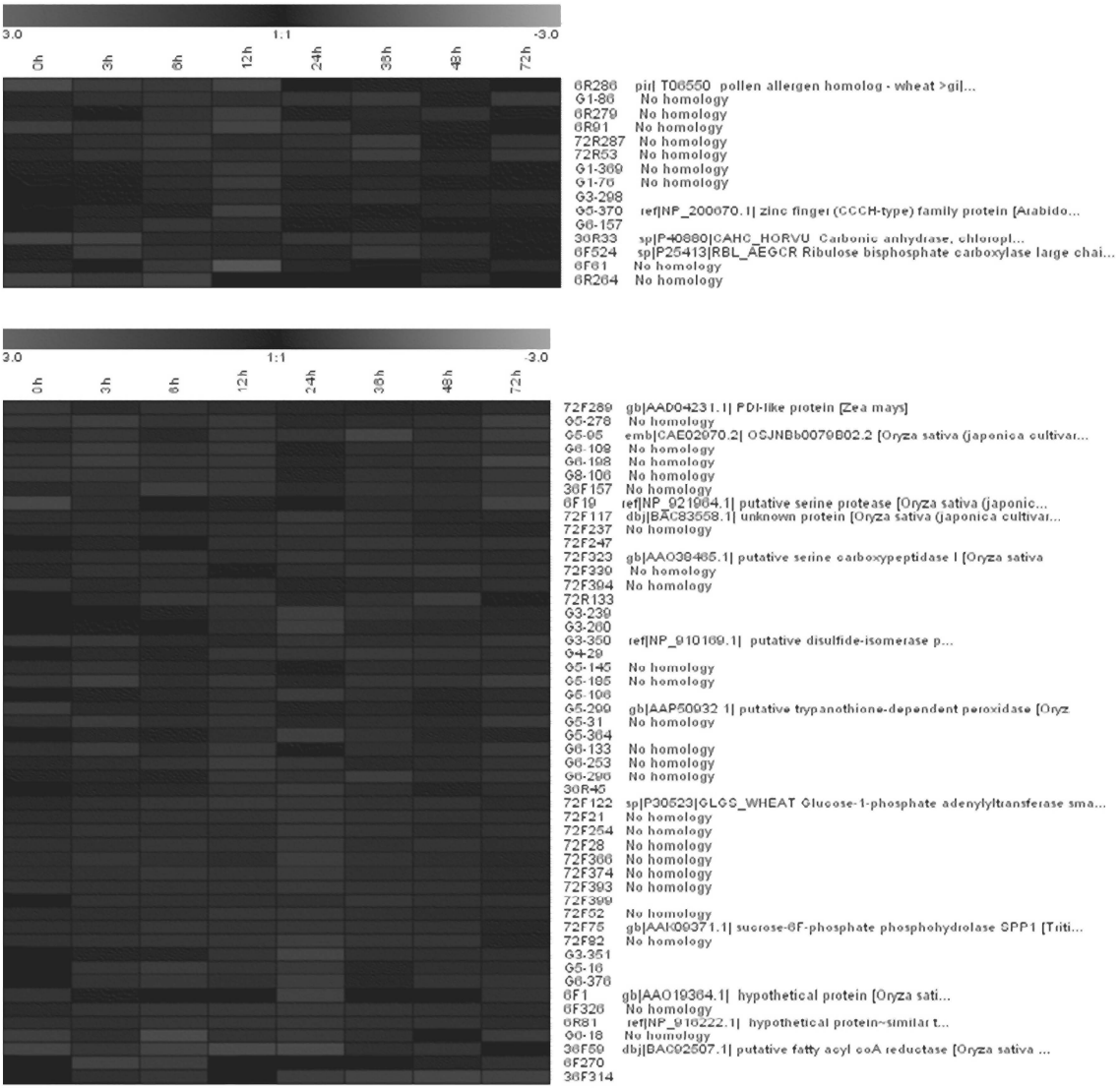


Figure 2.9. Clusters 9 and 10 of the differentially expressed ESTs between Ning 7840 and Clark. Each row represents one EST and each column corresponds to a particular time point. The scale of expression ranges (log2) from 3.0 (bright red) to –3.0 (bright green). Red boxes denote ESTs with expression levels significantly higher in the resistant variety relative to the susceptible variety. Green boxes mean that that particular EST is down-regulated in Ning 7840 (relative to Clark) at that particular time point. The highest similarity score generated by BLAST was considered the best match and was used as the putative identity of corresponding ESTs.

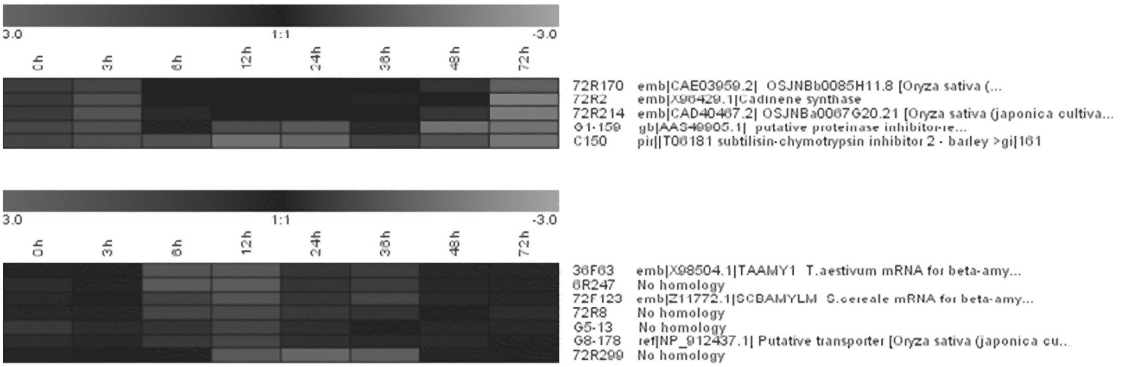


Figure 2.10. Clusters 11 and 12 of the differentially expressed ESTs between Ning 7840 and Clark. Each row represents one EST and each column corresponds to a particular time point. The scale of expression ranges (log2) from 3.0 (bright red) to –3.0 (bright green). Red boxes denote ESTs with expression levels significantly higher in the resistant variety relative to the susceptible variety. Green boxes mean that that particular EST is down-regulated in Ning 7840 (relative to Clark) at that particular time point. The highest similarity score generated by BLAST was considered the best match and was used as the putative identity of corresponding ESTs.

Figure 2.11. Mean expression level of 12 clusters of differentially expressed ESTs between Ning 7840 and Clark after fungal stress. The ESTs were grouped based on similarity of expression pattern (K-means) using Genesis software (Sturn et al, 2002). The X-axis represents the time point spikes were sampled while the y-axis shows

differential expression in log based two ratios. Gray lines represent individual ESTs and the pink line is the mean transcript abundance at the time course of infection.

CHAPTER III

DIFFERENTIALLY EXPRESSED ESTS

BETWEEN FHB RESISTANT AND SUSCEPTIBLE NILS

Introduction

In the previous chapter, 199 ESTs were identified as differentially expressed between Ning 7840 and Clark. Among the ESTs, some were identified as putative defense-related genes that may play important roles in FHB resistance. To further extend the search for key genes influencing FHB resistance, near isogenic lines (NILs) contrasting in FHB resistance were examined. Theoretically these lines should have similar genetic background except for the resistance gene(s) and as such may lead to the identification of type II FHB resistance genes.

Materials and Methods

NILs were derived from the cross of Ning 7840 x IL897978 (FHB susceptible), provided by Dr W-Z. Zhou from University of Illinois. The NILs were selected from within family segregation of F5 progenies. The resistant NIL showed a high level of Type II resistance with about 10% infected spikelets in an inoculated spike and the susceptible NIL showed almost 100% infected spikelets in an inoculated spike. Molecular marker analysis for the major QTL on 3BS showed that these NILs are polymorphic for marker alleles flanking the QTL. Based on previous experiments, 3, 12, 36 and 72 hai appeared to be important time points to detect differentially expressed ESTs, therefore *F. graminearum*-inoculated spikes of resistant and susceptible NILs were sampled at these time points for this experiment. Microarray hybridization, slide scanning and data analysis were done as described in Chapter II. An EST with a B value of at least 5 and a 1.5 times difference in fold expression (compared to the susceptible control) was considered significantly differentially expressed.

Real-time PCR

The same batch of RNA used for the microarray analysis was used for real-time PCR. First-strand cDNA synthesis was performed in a 24 µl reaction by combining 10 µg total RNA with 1 pmole oligo$(dT)_{12\text{-}18}$ (Invitrogen, Carlsbad, CA) and random primers (Promega Corporation, Madison, WI). The mix was incubated at 80°C for 10 min before transferring to an ice tub. Twenty units of SUPERaseIn Rnase Inhibitor (Ambion Inc., Austin, TX) was added to the RNA-primer mix. A 50 µl final reaction consisting of 10 µl 5X Superscript II first strand buffer, 3 ul of 10 mM dNTPs, 5 µl 0.1 M dithiotreitol, 200 U Superscript II enzyme (Invitrogen, Carlsbad, CA) and the RNA-primer mix was incubated at 42°C for 2 h. Real time-PCR (RT-PCR) was performed in an iCycler PCR machine (Bio-Rad Laboratories, Hercules, CA) with a 25 µl total reaction volume made up of 12.5 µl 2X iQ Sybr Green Supermix (Bio-Rad Laboratories, Hercules, CA), 1 µl each of 10 mM forward and reverse primers and 10.5 µl cDNA sample. All cDNA templates were diluted with 100 ul distilled water and amplified with ß-actin primers to check for differences in initial cDNA concentration. The templates were further diluted 10X and real time-PCR was conducted with actin as control and two technical replicates for each biological sample and treatment combination. The primers used to quantify gene expression were designed with Beacon Designer Software (Premier Biosoft International, Palo Alto, CA) and listed in Table 3.1. PCR amplification conditions consisted of an initial denaturation step at 95°C for 5 min followed by 45 cycles of denaturation at 95°C for 20 sec and annealing and extension at 60°C for 1 min. After amplification a melting step was performed consisting of 95°C for 1 min, cooling to 55°C for 1 min followed by a slow rise in temperature to 95°C at a rate of 0.5°C/ 10 sec.

Results

A portion of a microarray hybridized with cDNAs from resistant and susceptible NILs sampled at 36 hai is shown in Figure 3.1. A total of 77 ESTs were identified as differentially expressed between the resistant and susceptible NILs and 47 were from the SSH libraries derived FHB stressed wheat spikes and 30 (39%) were from SSH libraries derived from aluminum-stressed roots. The ratio between FHB/Al clones that were identified as differentially expressed in this study is consistent with that from the previous chapter. Differentially expressed genes were grouped into eight clusters (Figures 3.2 and 3.3) based on similarities in expression patterns according to K-means method using the Genesis software (Sturn et al. 2002).

Quantitative RT-PCR on resistant and susceptible cDNA templates was done using ß-actin primers (Figure 3.2). Results show that cDNA template concentrations across all time points in both resistant and susceptible NILs were equal. Moreover, these findings imply that expression of ß-actin is consistent and equal among all samples and is suitable to use as non-regulated control. RT-PCR was done to measure steady-state mRNA levels of two ESTs, 6F447 and 72R299, whose sequences were obtained from early sequencing results. For clone 6F447, the fold-change in gene expression level obtained from real time PCR was greater than that from the microarray data (Table3.2). However, the values obtained still confirm that the EST is not differentially expressed at 3-12 hai and down-regulated from 36-72 hai. The level of transcript alterations obtained for clone 72R292 using real time-PCR was also similar to the microarray data except for 3 hai. Results also confirm the down-regulation of this EST at 36-72 hai (Figure 3.3).

Based on limited data from real-time PCR, data from microarray correlates well with that obtained from RT-PCR (Table 3.2).

Photosynthesis-related ESTs

This group includes ESTs from clusters 2 and 7. Six ESTs in cluster 2 are down-regulated at 0 h but up-regulated from 3 to 12 hai. Three of them had similarity to Ribulose bisphosphate carboxylase (Rubisco), senescence associated protein and an unknown protein, while three others had no sequence similarity to known proteins in the database. This is the only cluster showing up-regulation of ESTs at early time points between NILs.

The general pattern of EST expression in cluster 7 is that they are down-regulated at 72 hai although up-regulation was observed in some ESTs at 3 and 12 hai. Three ESTs in this cluster had sequence similarity with the photosynthesis-related enzymes Rubisco activase and carbonic anhydrase. Another EST in this cluster was putatively identified as β-glucanase. Six other ESTs either corresponded to unknown proteins or had no significant similarity to known genes in GenBank.

Defense-related ESTs

This group includes clusters 3 and 4. ESTs in cluster 3 were down-regulated at 0 h and from 36 to 72 hai. They included putative UDP-glucose pyrophosphorylase, wheat pollen allergen, pathogen-related protein, two unknown proteins, and four ESTS without any hit in BLASTX search. The putative pollen allergen homolog and three unknown proteins (clones 6R256, 6R222) were up-regulated at 12 hai. Cluster 4 contains only five ESTs. Among them, one EST was similar to defense-related PAL, three ESTs were

identified as a putative giberellin 20-oxidase, phospho-2 dehydro-3-deoxyheptonate aldolase (DHAP) and a high affinity potassium transporter, and one EST did not have homology in GenBank. ESTs in this cluster are down-regulated at 36 hai.

Stress-related ESTs

There are 18 ESTs in cluster 5 and they are up-regulated at 36 to 72 hai. Among them are ESTs putatively identified as a peptidylprolyl isomerase, beta-amylase, SGT1 and heat shock proteins. Eight ESTs produced no hit during BLAST sequence homology search while three others corresponded to unknown/hypothetical proteins.

Other down-regulated ESTs

This group consisted of clusters 1, 6 and 8. Down-regulation is a predominant response to fungal stress in at least one time point. The ESTs in the first cluster were mainly initially up-regulated at 0 h but down-regulated in most time points after fungal stress, especially at 36 to 72 hai. ESTs in this cluster had sequence homology with peptidase M48, MRP-like ABC transporter, fatty acid CoA reductase and P450. Five out of nine ESTs in this cluster either correspond to unknown proteins or do not have sequence similarity with known genes.

The nine ESTs in cluster 6 are generally down-regulated at 3 hai and differential expression is not obvious after 3 hai for most of the ESTs. ESTs in this cluster showed homology with genes involved in cell wall degradation, membrane transport and chlorophyll biosynthesis.

Cluster 8 consists of seven ESTs. Two of them had sequence homology with a putative calreticulin and a citrate synthase. They were all down-regulated at most of the

time points from 0 to 36 hai, but a consistent high level of down-regulation occurred at 36 hai. Differential expression was not observed at 72 hai.

Among the 77 differentially expressed genes identified, 22 were also found as differentially expressed between Ning 7840 and Clark (Table 3.3). Four of them were from the libraries derived from aluminum-stressed wheat roots.

Discussion

Differentially expressed ESTs in NILs

A total of 77 differentially expressed ESTs were identified between *F. graminearum*-inoculated resistant and susceptible NILs, which is about one third of differentially expressed ESTs between two varieties as described in the previous chapter. The result indicated that two-thirds of ESTs were eliminated when NILs were used instead of the two varieties. Some of these eliminated ESTs may be related to different genetic backgrounds between the two varieties. One-third of the ESTs identified in this experiments were also identified in the previous experiment, but other ESTs, which were not detected in the previous microarray experiment, were also identified in this study. This may be due to NILs were derived from different genetic backgrounds other than Clark, the susceptible variety used in the previous study, because most of differentially expressed genes were down-regulated. In addition, two NILs may not be perfectly isogenic and other genetic differences may still exist between the two NILs. The large number of differential expressed genes identified between NILs suggests the complexity of FHB resistance.

Of the 77 differentially expressed ESTs in this study, 47 were from a FHB-stressed library and 30 (39%) were from Al-stressed libraries. Among 22 commonly expressed ESTs between NILs and the two varieties, 18 were from the FHB stressed libraries and four (18%) from aluminum-stressed root libraries. The results show that the majority of the significantly differentially expressed ESTs were from the FHB libraries.

Defense-related ESTs

In this experiment, general-defense related genes such as PR-1, chitinase, TLPs were not differentially expressed during early time points after fungal inoculation, which is different from the result in the previous chapter. This may suggest that general defense-related genes induced by *F. graminearum* may not be necessary for wheat type II resistance to FHB.

This study demonstrated that the expression level of stress-related ESTs was mainly up-regulated at 72 hai with some of them also up-regulated at 36 hai (cluster 5). This agrees with Pritsch et al. (2001) that fungal spread within a spike of Sumai 3 (source of Ning 7840 resistance genes) might occur after 48 hai. Two ESTs were putatively identified as SGT1 and cytosolic heat shock protein (HSP 90) in barley. In addition, heat shock protein 80 is a homolog of cytoplasmic heat shock protein 90. SGT1 is essential for the activation of some resistance genes or defense signaling (Austin et al. 2002; Glazebrook 2001; Feys and Parker 2000). SGT1 is a positive regulator of the SCF E3 ubiquitin ligase complex, a complex involved in protein degradation. Up-regulation of SGT1 suggests that it may be involved in the degradation of a negative regulator of the FHB disease resistance pathway. Both SGT1 and HSP90 have been reported to interact with each other and are involved in RPS2-mediated disease resistance in *Arabidopsis*

(Takahashi et al. 2003). The EST putatively identified as SGT1 is from an FHB-stressed library while the heat shock proteins are from the aluminum-stressed library. In barley, SGT1 was shown to interact with RAR1, an essential component of a signaling pathway common to many R genes (Azevedo, 2002). SSH is a technique that removes common sequences between two populations. However, if a sequence differs by only a point mutation between the tester and driver population, it may probably be subtracted out. Thus, some potentially useful and important ESTs may have been removed. This could be a reason why an EST corresponding to RAR1 was not identified in this study. Alternatively, SGT1 probably interacts with some other genes such as the unknown protein or four other ESTs without homology to known genes from the same cluster.

Other ESTs

Photosynthesis related genes were differentially expressed in the resistant RIL soon after the fungus contacts the wheat tissue (from 3 to 12 hai). Studies by Somssich and Halbrock (1998) reported the inverse relationship between expression of photosynthesis-related and defense-related genes. When defense related genes are expressed, photosynthesis is down-regulated presumably to re-allocate carbon flux toward defenses. Therefore, decreasing the expression level of photosynthesis genes for several hours may enhance FHB resistance in wheat spike.

ESTs (from an aluminum-stressed root library) with similarity to protein disulfied isomerase (PDI) and heat shock-inducible peptidylprolyl isomerase (PPI) were also up-regulated. PDI catalyzes the folding of disulfide-bonded proteins (Klappa et al. 1997). This gene was also up-regulated in wild rice infected with *Magnaporthe grisea* (Shim et al. 2004). On the other hand, PPI accelerates protein folding during protein synthesis

(Miernyk, 1999). *F. graminearum* produces a mycotoxin that inhibits protein synthesis (Kimura et al. 1998). DON was detected in *F. graminearum*-inoculated wheat spikelet as early as 48 hai (www.btny.purdue.edu/NC129/report98.html). However, up-regulation of PPI suggests that protein synthesis is not inhibited in the resistant NIL at 72 hai. Other up-regulated ESTs from the Al-stressed root libraries were four ESTs with no significant similarity with genes in GenBank. These EST are probably stress-related and not specific to FHB resistance.

Common up-regulated ESTs in resistant NIL and Ning 7840

Two ESTs that had sequence similarity with the photosynthesis-related enzymes Rubisco and carbonic anhydrase were up-regulated at 3-12 hai. Carbonic anhydrase converts bicarbonate ions to carbon dioxide for photosynthesis (Lindskog et al., 1971). On the other hand, Rubisco is an enzyme involved in carbon fixation (Oneal et al. 1987). Another up-regulated EST at 12 hai corresponded to an unknown protein. This EST may also be involved in photosynthesis based on cluster analysis. These ESTs may not be directly involved in FHB resistance, but photosynthesis is obviously affected by *F. graminearum* infection.

An EST with sequence homology to a wheat pollen allergen homolog was also up-regulated at 12 hai. It was observed that *F. graminearum* grows abundantly on pollen surface (Ribichich et al. 2000). Moreover, a study by Naik and Busch (1978) showed that pollen extracts from corn increased germ tube length and germination rates of *F. graminearum*. The biological significance of the induced expression of this EST in resistant genotypes is unknown and needs further investigation.

Common down-regulated ESTs in resistant NIL and Ning 7840

One EST having similarity with P450 71C4 is involved in the synthesis of 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), a major anti-microbial compound found in cereals. DIMBOA inhibits proteases and oxidative enzymes in fungi, bacteria and insects (Niemeyer, 1988). Among the ESTs with similarity to known genes in GenBank, this seems to be the only one that is defense-related.

ESTs with similarity to putative giberellin 20-oxidase and a putative high affinity potassium transporter were down-regulated at 36 hai. Gibberellic acid is a hormone that is involved in many aspects of plant development including control of flower formation and development and fruit setting (Kang et al. 1999). Down-regulation of this EST is probably related to the plant's development and not to FHB resistance. On the other hand, high affinity K+ transporter plays a role in the uptake of K^+ from the soil (Santa-Maria et al. 1997). Studies by Kruger et al (2002) also identified an EST with similarity to a high-affinity potassium transporter from a Sumai 3 library made from wheat spikes 24 h after spray inoculation with *F. graminearum*. This transporter may play a role in FHB resistance. These common down-regulated ESTs need further investigation to determine their role in FHB resistance.

ESTs that exhibited different expression patterns between NILs and Ning/Clark

ESTs that had similarity with beta-amylase, putative calreticulin, peptidase M48 exhibited different expression profiles compared to the results in Chapter II. In addition, one unknown protein and two ESTs with no BLAST hit from the FHB libraries as well as four ESTs from aluminum stressed libraries belong to this group. The difference in the genetic composition of the susceptible control used in the two microarray experiments

may be one of the reasons for the observed discrepancy. The up- or down-regulation of an EST in the resistant line/variety is measured relative to transcript abundance of the susceptible control. Since the susceptible controls are genetically different, different patterns of down-regulation will be observed. Expression of these ESTs during fungal stress may just be a consequence of disease resistance and are not the FHB resistance genes *per se*. NILs contrasting in FHB resistance would theoretically have the same genetic background and will only differ in the presence of a functional resistance gene(s). However, NILs are oftentimes not purely isogenic and some genetic differences not related to FHB may still be observed. In addition, we still cannot exclude the possibility that some of these genes may play a role in the resistance of wheat to FHB.

Quantitative real-time PCR analysis

Several studies have shown that data from real time PCR correlate well with microarray data (Kawasaki et al, 2001; Puthoff, 2003 and Ozturk, 2002), therefore real - time PCR is widely accepted as a useful means to validate microarray results. In this study, real-time PCR was performed for two ESTs (72R292 and 6R447) to validate the microarray result. EST 72R292 is from the 72 FHB stressed library. Real-time PCR showed the down-regulation of this EST at 36 and 72 hai, which agrees with microarray result. The same result was obtained for EST 6R447 (Figure 3.3). However, a much larger increase in fold expression was obtained from real-time PCR than that from microarray analysis (Table 3.3). Microarrays have a relatively low dynamic range due to low concentrations of DNA deposited in slides (Ozturk et al, 2002). As such, they sometimes do not reflect the true changes in transcript abundance especially for strongly up- or down-regulated transcripts. In general, the results we obtained from microarray

correlated with the RT-PCR results. Verification of other differentially expressed ESTs will be done in the near future.

Table 3-1. Primers used in real time PCR

Name	Sequence	Length	Tm	GC%
72R292_F1	AGGGTATTCCGATGTGTGGTG	21	57	52.4
72R292_R1	TCAAGGGAATGACTACTTTCGC	22	55.9	45.5
6F447_F1	GGAAGTTGAGGGCGTGAAAG	20	56.1	55
6F447_R1	GGTACTAGCTGCAAGTCTGATG	22	56	50
actin_F1	CCTTCCACATGCCATCCTTC	20	55.6	50
actin_R1	GCTTCTCCTTGATGTCCCTTAC	22	55.9	50

Table 3.2. Gene expression in fold change from microarray and real-time PCR data

	72R292		6F447	
Time	Microarray	Real Time PCR	Microarray	Real Time PCR
3 hai	1.04	1.74	1.09	1.41
12 hai	1.06	1.23	1.03	1.27
36 hai	-2.9	-4.0	-2.22	-2.9
72 hai	-1.74	-1.68	-2.32	-32

Table 3.3. Differentially expressed ESTs between resistant and susceptible genotypes

ID	Putative ID	E value
36F150	dbj|BAB01742.1| unnamed protein product [A. thaliana]	3.00 E-05
36F63	emb|X98504.1|TAAMY1 T.aestivum mRNA for beta-amylase	7.00 E-58
36R135	dbj|BAD08938.1| putative cytochrome P450 71C4 [O. sativa]	1.00 E-15
36R33	sp|P40880|CAHC_HORVU Carbonic anhydrase, chloroporoplast	9.00 E-42
36R353	ref|NP_915149.1| putative calreticulin [O. sativa]	1.00 E-26
6F420	gb|AAS88823.1| unknown protein [Oryza sativa]	5.00 E-48
6F427	emb|CAA25058.1| ribulosebisphosphate carboxylase [T. aestivum]	4.00 E-14
6R130	ref|NP_199987.2| peptidase M48 family protein [A. thaliana	5.00 E-11
6R247	No homology	
6R264	No homology	
6R279	No homology	
6R286	pir|T06550| pollen allergen homolog - wheat	2.00 E-24
72F123	emb|Z11772.1|SCBAMYLM S.cereale mRNA for beta-amylase	1.00 E-33
72R162	gb|AAO00709.1|Putative gibberellin 20-oxidase [O. sativa]	2.00 E-23
72R198	No homology	
72R275	No homology	
72R292	ref|NP_914903.1| putative high-affinity potassium transporter [O. sativa]	4.00 E-60
72R8	No homology	
C124	No homology	
G3-273	gi|33147060|dbj|BAC79963.1| unknown protein [O. sativa]	6.00 E-07
G5-37	dbj|BAB03377.1| unnamed protein product [O. sativa]	6.00 E-21
G8-34	No homology	

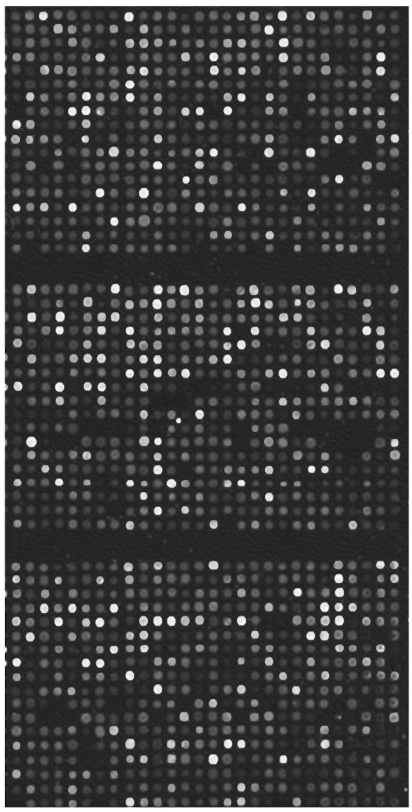


Figure 3.1. A pseudocolor image of a portion of a dye-flip slide hybridized with cDNAs from resistant and susceptible NILs sampled at 36 hai. To generate this image slide-bound cDNA were hybridized with cDNAs from wheat spikes sampled at 36 hai with the pathogen. Complementary DNAs from resistant and susceptible lines were labeled with cy3 and cy5, respectively. Up-regulated ESTs in the resistant line are shown as green spots, down-regulated ESTs as red, and yellow spots correspond to non-differentially expressed ESTs between the resistant and susceptible lines.

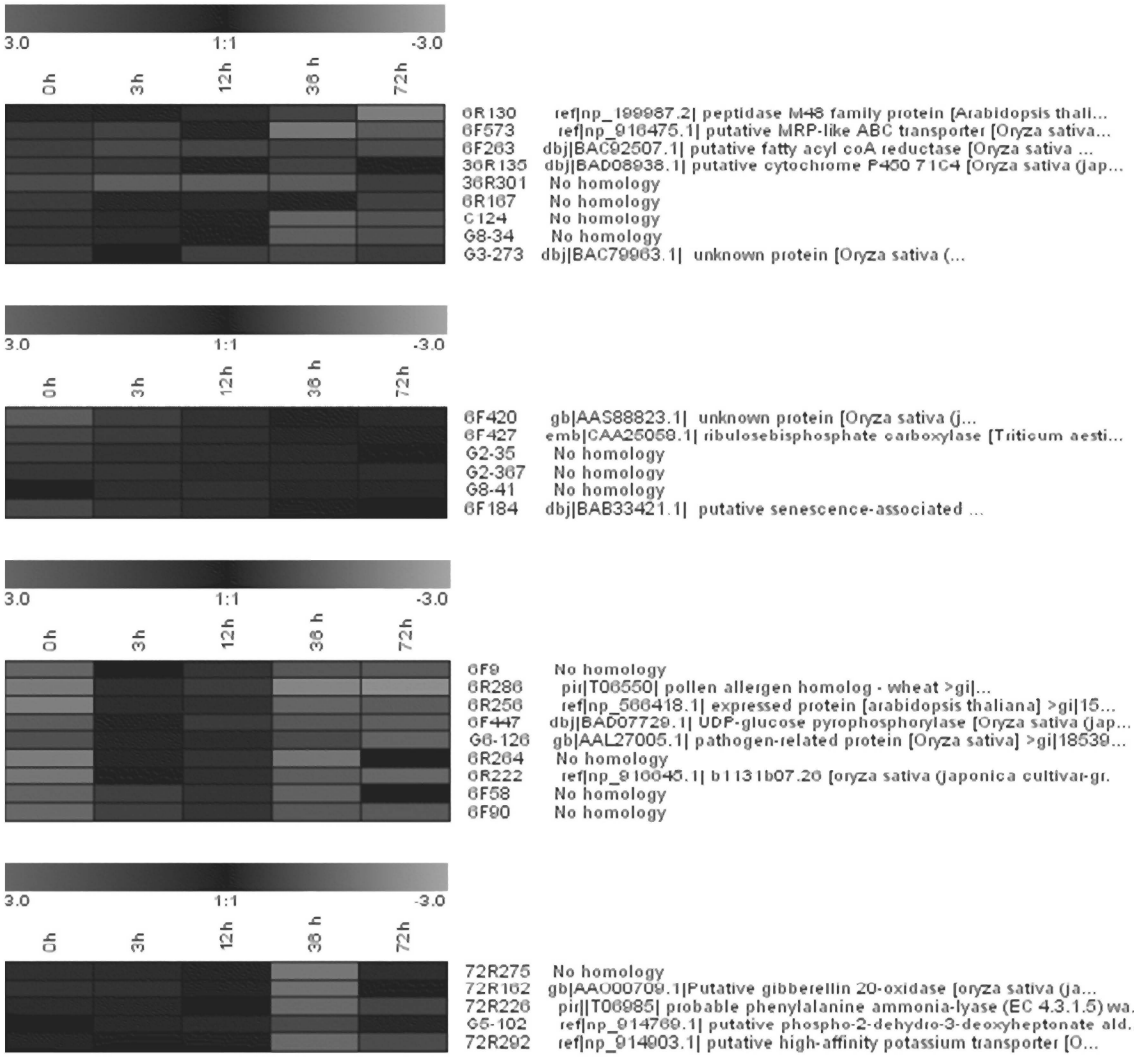


Figure 3.2. Cluster 1 to 4 of the differentially expressed ESTs between resistant and susceptible NILs. Each row represents one EST and each column corresponds to a particular time point. The scale of expression ranges from 3.0 (bright red) to –3.0 (bright green). Red boxes denote ESTs with expression levels significantly higher in the resistant line relative to the susceptible line. Green boxes mean that that particular EST is down-regulated in the resistant NIL (relative to the susceptible NIL) at that particular time point. The highest similarity score generated by BLAST was considered the best match and was used as the putative identity of corresponding ESTs.

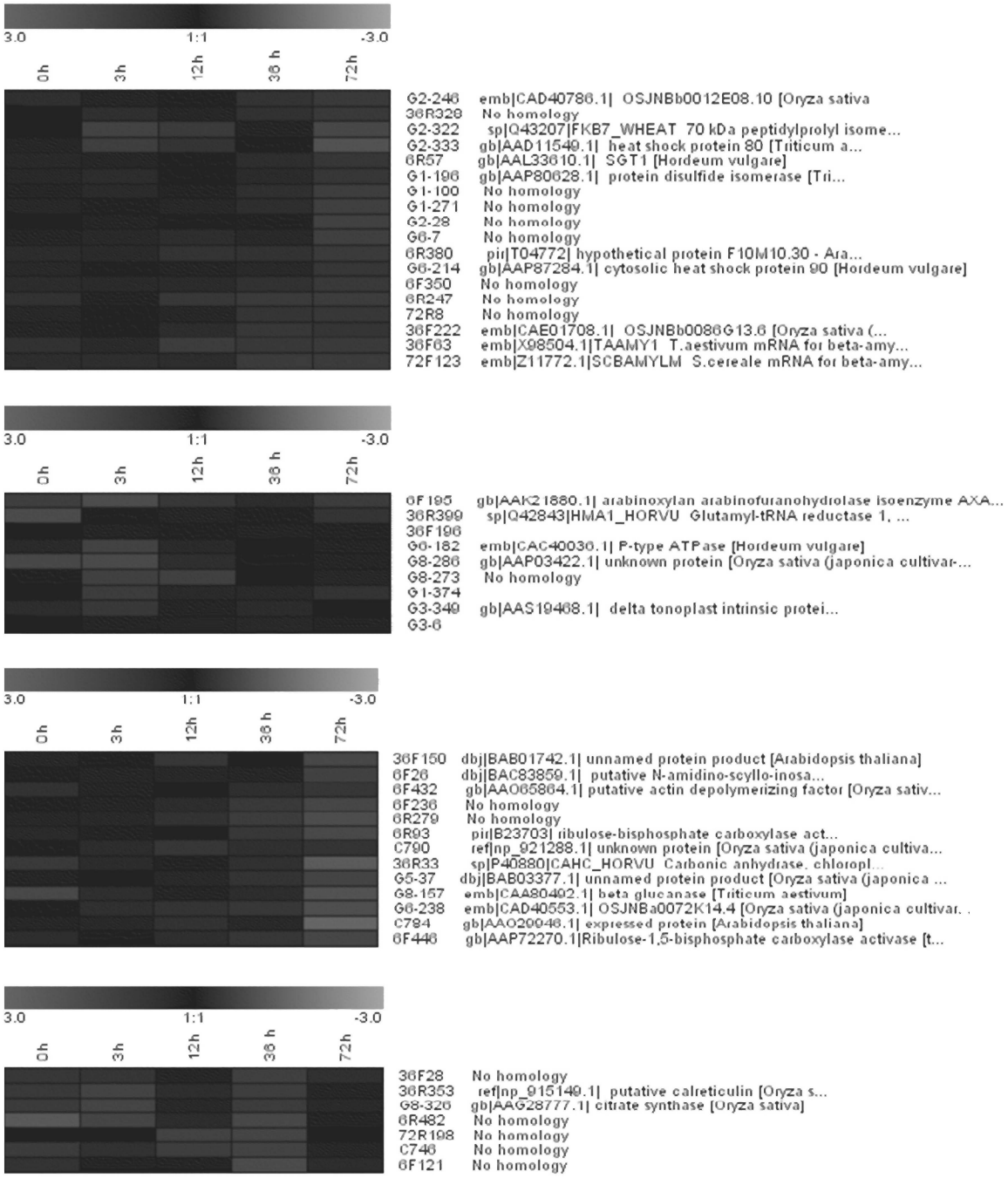


Figure 3.3. Cluster 5 and 8 of the differentially expressed ESTs between resistant and susceptible NILs. Each row represents one EST and each column corresponds to a particular time point. The scale of expression ranges from 3.0 (bright red) to –3.0 (bright green). Red boxes denote ESTs with expression levels significantly higher in the resistant line relative to the susceptible line. Green boxes mean that that particular EST is down-

regulated in the resistant NIL (relative to the susceptible NIL) at that particular time point. The highest similarity score generated by BLAST was considered the best match and was used as the putative identity of corresponding ESTs.

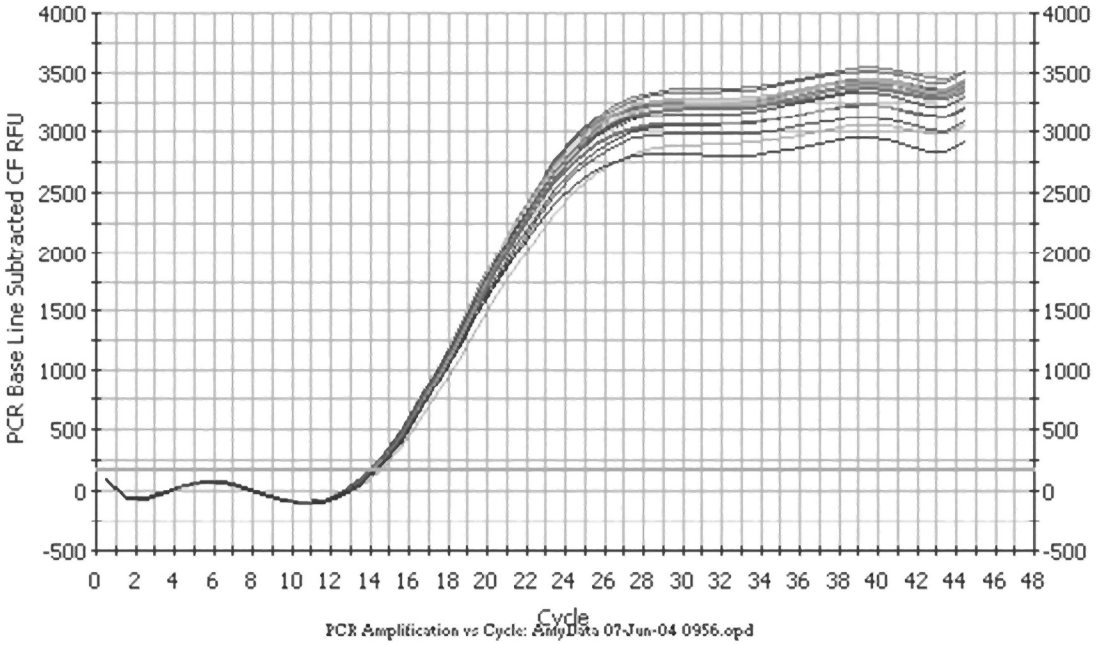


Figure 3.4 Fluorescence signal of eight cDNA templates amplified with wheat beta-actin primers (non-regulated control) indicate equal template concentrations.

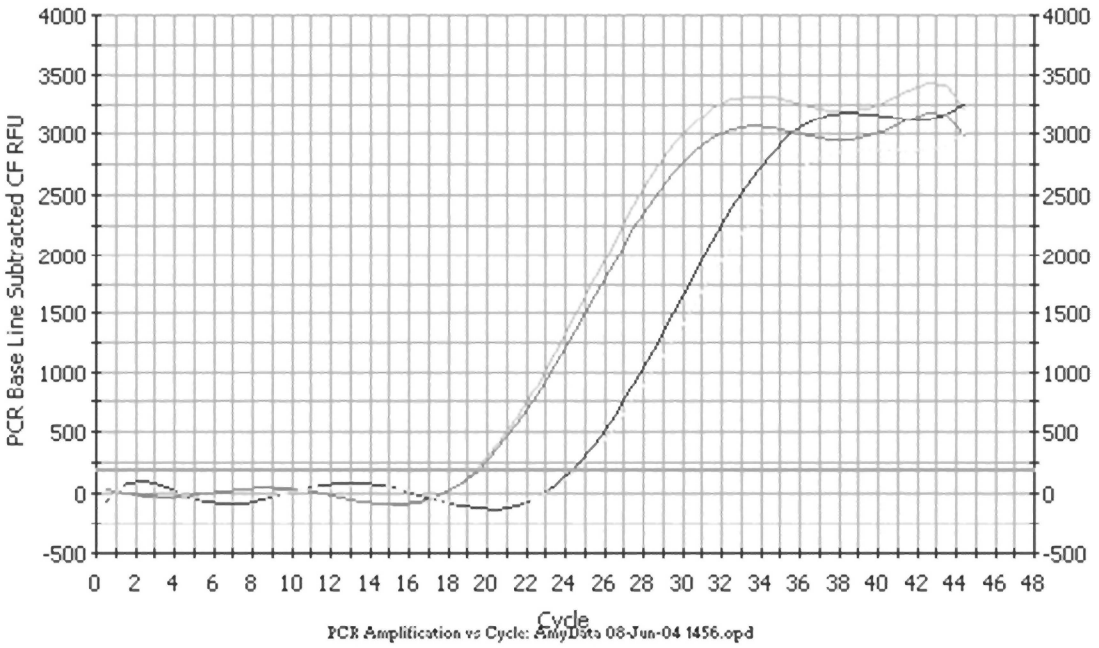


Figure 3.5. Gene expression level of 6F447 for the 72 hai susceptible and resistant NILs. The fluorescence signal of the target gene at each cycle is shown as light blue and pink lines in the susceptible and dark blue and yellow in the resistant NILs. Earlier amplification was observed in the susceptible NIL implying higher gene expression compared to the resistant.

CHAPTER IV

FUNGAL-INDUCED ESTS IN NING 7840 AND CLARK

Introduction

Plants cope with pathogen attack by triggering a set of defense responses. Host-pathogen interaction is often a complex, dynamic system that involves an integrated set of genes that is difficult to monitor using conventional genetic and biochemical methods. DNA microarrays allow the simultaneous analysis of thousands of genes in one experiment (Freeman et al. 2000; Hedge et al. 2000). It enables the detection and identification of up- or down-regulated genes. Schenk et al (2000) compared the expression profile of Arabidopsis infected with *Alternaria brassicicola* to uninfected controls using an array with 2,375 genes. They were able to identify 168 induced and 39 repressed genes in response to fungal infection. A study by Ramonell (2002) identified 61 differentially expressed genes in Arabidopsis by comparing chitin-treated and untreated plants. These studies demonstrate the usefulness of microarrays in identifying genes that are differentially expressed between treated and untreated plants.

In Chapter II, 199 significantly differentially expressed ESTs were identified between Ning 7840 and Clark. By directly comparing fungal-inoculated resistant and susceptible varieties, we were able to determine the relative abundance of fungal resistant transcripts relative to the susceptible control. However, information on dynamic changes of differentially expressed genes due to fungal infection in two wheat cultivars with contrasting FHB resistance may show a whole picture on how wheat responds to FHB infection, provide insight into wheat defense related pathways, help to understand mechanism of wheat resistance to FHB, and separate fungal induced genes from constitutively expressed genes. Therefore, the objective of this chapter is to identify the

ESTs that were differentially expressed between fungal-inoculated and mock-inoculated resistant and susceptible cultivars during early fungal infection events.

Materials and Methods

Inoculated spikes of Ning 7840 and Clark were sampled at 6, 36 and 72 hai with *F. graminearum*. Plant inoculation, RNA extraction, microarray hybridization, slide scanning and data analysis were conducted as described in Chapter II. For this experiment, an EST with a B value of at least 2 and a 1.5 times difference in fold expression (compared to the susceptible control) was considered significantly differentially expressed. B value was lowered because of fewer replicates.

Results

A portion of a microarray hybridized with cDNAs from fungal-inoculated and mock-inoculated Ning (A) and Clark (B) sampled at 36 hai is shown in Figure 4.1.

Fungal-induced ESTs in Ning 7840

Eighteen ESTs in Ning 7840 were differentially expressed after inoculation with *F. graminearum* (Table 4.1). Among them, 11 ESTs were from the FHB libraries and seven were from the aluminum-stressed wheat root libraries. These observations show that some wheat genes that respond to fungal infection may also be induced by aluminum stress. Seven ESTs were significantly up-regulated at 6 hai, indicating that a higher level of expression of these ESTs in Ning 7840 was induced by the fungal stress. Among them, four ESTs had similarities to a putative proteinase inhibitor-related protein, β-glucanase, long-chain fatty acid CoA ligase and UDP-glucose pyrophosphorylase, respectively; two (clones 6R256 and 6R347) had homology with unknown proteins; while another clone

(6R264) did not produce any hit in the GenBank. Two down-regulated ESTs, whose expression was suppressed by fungal stress in Ning 7840 at 6 hai, were an EST with homology to a barley thiol protease precursor and an EST (G3-305 from aluminum library) that had no significant similarity with any gene in the GenBank. At 36 hai, four genes were significantly up-regulated and only 72R299 with no similarity to known genes in the GenBank was down-regulated. Among the up-regulated ESTs, one had similarity to an unknown protein, two did not have similarity to known genes, and another one had no successful sequence. Four ESTs were differentially expressed at 72 hai. Two up-regulated ESTs were similar to P450 and phenylalanine ammonia lyase (PAL), and another up-regulated EST and down-regulated EST had no similarity to any gene in the GenBank.

Fungal-induced ESTs in Clark

A total of 86 ESTs were significantly differentially expressed between fungal inoculated Clark and control at 36 and 72 hai (Tables 4.2, 4.3 and 4.4). Sixty three percent of induced clones came from the FHB stressed libraries. No differentially expressed ESTs were identified at 6 hai.

The number of down-regulated ESTs, whose expression was repressed by fungal infection, at 36 and 72 hai was 24 and 6, respectively. ESTs that were down-regulated at 36 hai had homology to genes involved in photosynthesis, biosynthesis of sucrose and fatty acid, starch metabolism and transport facilitation. ESTs with similarity to beta-amylase, copper chaperone, protein disulfide isomerase and glucose-1-phosphate adenylyl cyclase were down-regulated at 72 hai.

Forty-seven ESTs were up-regulated (expression induced by *F. graminearum*) in the susceptible cultivar Clark at 36 hai. Among them, two ESTs, protein phosphatase 2C and a putative serine/threonine kinase receptor precursor, may be involved in signal transduction and six ESTs have sequence similarity to defense-related genes such as PR-proteins and other anti-microbial compounds.

There were 35 up-regulated ESTs at 72 hai. Twenty-one of them were also up-regulated at 36 hai. Seven ESTs induced at 72 hai have similarity to high-affinity potassium and PDR-like ABC transporters, P450s, PAL and PR-proteins, respectively. Results showed that far more genes were differentially expressed in Clark than that in Ning 7840.

Eight ESTs induced in Clark were also induced in Ning 7840. Among them, three ESTs had similarity with P450, proteinase inhibitor and long chain fatty acid CoA ligase, one corresponded to an unknown protein and four other ESTs had no homology to other known genes.

Discussion

Microarray analysis was conducted using fungal and mock-inoculated spikes from 6, 36 and 72 hai. Several studies have shown that the *Fusarium* macroconidia germinates after 6 hai, directly enters the top part of the ovary, inner lemma and inner palea 36 hai and spreads after 48 hai (Pritsch et al. 2000 & 2001; Kang and Buchenauer, 2000). These time points reflect important fungal activities and were chosen. Using an array printed with clones from SSH libraries, 18 and 86 transcripts with altered mRNA levels (compared to mock-inoculated controls) were identified in Ning 7840 and Clark, respectively. The number of differentially expressed ESTs is probably lower than

expected. The SSH libraries in this study were made from *F. graminearum*-inoculated resistant and susceptible RILs. Theoretically, any gene that was commonly induced as a response to fungal stress was subtracted out. Thus, the library may only contain a subset of fungal-induced ESTs. This may lead to a significant decrease in number of fungal-induced genes. In addition, we only found half as many differentially expressed genes in this study compared to Chapter II even after lowering the cut off B value to 2. The results suggest that many ESTs differentially expressed between Ning 7840 and Clark may be constitutively expressed. ESTs from the aluminum-stressed libraries (39%) were also induced by *F. graminearum.* This suggests that these fungal-induced genes are probably related to general defense and can also be induced by abiotic stresses.

Defense-related ESTs

An EST with similarity to a putative proteinase inhibitor-related protein was up-regulated by at least two-fold in Ning 7840 at 6 hai. Studies conducted by Dunaevskii et al. (1997) showed that trypsin/chymotrypsin (a proteinase inhibitor) inhibits germination and growth of *Alternaria alternata* in buckwheat. Studies by Cordero et al. (1994) demonstrated the up-regulation of a proteinase inhibitor gene after wounding and fungal infection. Moreover, Richards et al. (1998) reported that a high level of aluminum induced expression of proteinase inhibitors. Proteinase inhibitors also disrupt the protein metabolism of insects that may attack the plant after a plant is wounded (Hammond-Kosack and Jones, 2000). The proteinase inhibitor related-protein was up-regulated at 6 hai presumably to prevent germination and growth of *F. graminearum.*

In Ning 7840, high transcript accumulation of secondary metabolites (relative to mock-inoculated) occurred at 72 hai. ESTs with similarity to P450 and PAL were

induced as a response to early fungal infection. The wheat P450 gene belongs to the CYP71C subfamily and encodes for an enzyme that participates in the synthesis of 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) (Frey et al. 1997). DIMBOA inhibits proteases and oxidative enzymes in fungi, bacteria and insects (Niemeyer, 1988). PAL is a key enzyme in the phenylpropanoid pathway and this pathway is involved in the production of defense-related secondary metabolites such as salicylic acid, phytoalexins, and lignin-like polymers (Hahlbrock and Scheel, 1989). Our result is in parallel with the study by Ye et al. (1990) showing that the specific activity of PAL increased in wheat spikes 2-4 days after inoculation (relative to uninoculated control). They further reported the decrease in enzyme activity from 4-8 days in resistant variety Sumai 3. Up-regulation of these two defense-related ESTs coincided with the down-regulation of Rubisco at 72 hai. This is consistent with observations from previous chapters that up-regulation of defense-related ESTs coincides with down-regulation of photosynthesis-related ESTs.

A high level of induction of chitinase, TLP, PR1-a and alternative oxidase was observed at 36 hai in Clark but not in Ning 7840. These ESTs are fungal-induced and not constitutively expressed. ESTs with similarity to PR1-1 and P450 were also up-regulated at 72 hai. This observation parallels the finding of Li et al. (2001) that transcripts of PR-proteins accumulate as early as 24 hai in susceptible mutants of Sumai 3. This is also consistent with results of the down-regulation of these ESTs in Chapter II. In addition, these observations further supports our finding in chapter II that defense response is induced earlier in Ning 7840 compared to Clark. Results from the both chapters imply that up-regulation of defense-related ESTs in Ning 7840 occurs before or around the time

of *F. graminearum* germination whereas in Clark, defense response occurs when the pathogen penetrates floral tissues.

Other ESTs

The most strongly up-regulated EST in Ning 7840 (6F447) was putatively identified as UDP-glucose pyrophosphorylase. This enzyme catalyzes the conversion of glucose-1-phosphate to UDP-glucose, a primary metabolite for carbohydrate metabolism. UDP-glucose is used in the production of cellulose and callose (Gibeaut, 2000) which can reinforce cell walls to prevent or reduce fungal penetration. Induction of this EST coincides with the time of *F. graminearum* germination as reported by Pritsch et al (2000). This implies that the plant senses the presence of the pathogen even before fungal penetration. Moreover, the strong induction of this EST suggests that modification of plant cell walls may be a key initial defense response against *F. graminearum.* This EST was not identified as differentially expressed in Chapter II. The up-regulation of transcripts that have DNA sequence similarity with genes implicated in signal transduction (protein phosphatase 2C and a putative serine-threonine kinase receptor protein) was observed in Clark at 36 hai. Cheong et al. (2002) also reported the induction of these genes in Arabidopsis as a response to wounding and pathogen infection. Like the UDP-glucose pyrophosphorylase, these ESTs were also not identified as differentially expressed in Chapter II. One possible reason could be that 6F447 was differentially expressed in Ning 7840 and the kinase and phosphatase receptor in Clark, but they did not express strong enough to meet the more stringent criteria we used in Chapter II

Two ESTs in Clark having sequence similarity to P450 CYP51 gene (clones 72R197) and a putative 3-beta hydroxysteroid dehydrogenase/isomerase (72R321) were

up-regulated relative to mock-inoculated susceptible control. These genes are potentially involved in sterol biosynthesis. Although the function of CYP51 gene in rice is unknown, the involvement of CYP51 in tobacco sterol biosynthesis has been reported (Burger et al, 2003). These compounds are very critical for the proper function of proteins located on membranes (Hartmann, 1998). Other genes identified in Clark such as membrane-associated transporters and ion channels may be involved in transducing signals from hormones, pathogens, and various environmental stresses. For instance, the expression of NtPDR1 (PDR-like ABC transporter) gene in tobacco was induced by an elicitor suggesting the involvement of some PDR genes in plant defense (Sasabe et al., 2002). Another transporter, vacuolar ATP synthase subunit H, was up-regulated in Clark. This transporter is responsible for pumping protons into vacuoles and generating an electrochemical proton gradient across the membranes that can be utilized for the secondary transport of ions, and solutes (Sze et al., 1992).

Results show that the induction of ESTs putatively involved in signaling in Clark coincided with the time the pathogen penetrates the top of the ovary, inner palea and lemma. No EST putatively involved in signal transduction was identified in Ning 7840. The induction of transcripts involved in signaling is probably transient and was not captured by our chosen time points.

Comparison of fungal-induced ESTs in Ning 7840 and Clark

There were eight ESTs commonly induced in the resistant and susceptible varieties as a response to *F. graminearum* infection. Common EST refers to the same EST clone and not contigs. Four ESTs were induced at different time points between Ning 7840 and Clark (earlier in one or the other) suggesting that the ESTs were

differentially expressed between Ning 7840 and Clark at either 6, 36 or 72 hai and thus was not subtracted out during SSH. On the other hand, one clone (G6-222) is from the aluminum-stressed root library and as such is not surprising that it could be induced in both FHB resistant and susceptible varieties. One EST (6F88) with homology to P450 and another with no homology (G6-222) were induced earlier in Clark than in Ning 7840. The wheat P450 gene participates in the synthesis of the defense compound DIMBOA. This is a common defense-related gene in cereals and may be a general defense response gene.

On the other hand, a putative protease-inhibitor related-protein was induced earlier in the resistant plant than in the susceptible plant. The induction of this EST in Ning 7840 coincided with the time of germination of *F. graminearum* in wheat spikes and this may play an important role in the early defense response in the resistant variety. Fatty acid CoA ligase, an enzyme involved in fatty acid biosynthesis, was also induced earlier in the resistant variety than in the susceptible variety. The biological significance of this observation in relation to plant defense is unknown.

One EST (72R305) with no similarity to known genes in GenBank was induced in both resistant and susceptible varieties at 36 hai. Based on log2 ratios, clone 72R305 was more strongly up-regulated in Clark compared to Ning 7840. This transcript may have been present in abundance and could be the reason why it was not eliminated during SSH library construction. This EST was up-regulated in both varieties and are probably related to general plant defense.

Four ESTs that had opposite expressions in Ning 7840 and Clark were identified. Two unknown ESTs (6R347 and G3-12) and an EST with no significant similarity to

other known genes (72R53) were fungal-induced in the resistant variety but suppressed by the pathogen in the susceptible variety. Cluster analysis for fungal-induced transcripts in Ning 7840 and Clark was not done because data from only two or three time points will probably generate less meaningful clusters compared to data from eight time points (chapter II). Based on cluster data from the previous chapter, clone 6R347 may be putatively involved in photosynthesis because it belonged to a clustered with mostly photosynthetic-related ESTs. A transcript that did not produce any hit in the database (72R299) was up-regulated in Clark but down-regulated in Ning 7840. We cannot infer what putative function 72R53 and 72R299 may have based on cluster analysis results from the previous chapter. On the other hand, successful sequence information is not available for clone G3-12. Clones 6R347, 72R53 and G3-12 were induced at 36 hai in Ning 7840. In Clark, ESTs with homology to PR proteins and signal transduction genes were induced at this time point. These results imply that fungal penetration induces a different set of genes in Ning 7840.

The data obtained for clones 6R347, 72R53 and 72R299 are in agreement with results from the chapter II and confirms the repeatability and reliability of our microarray results. The differences in the induction or repression of 72R299, 72R53 and G-12 in the resistant and susceptible varieties make it tempting to presume that these genes may play an important role in FHB resistance.

In Ning 7840, up-regulation of defense-related ESTs occurred at 6 hai. This is in agreement with the early induction of defense-related ESTs observed in the previous chapter. In contrast, no significantly differentially expressed genes were observed in Clark at this time. A study by Li et al. (2001) showed that transcript levels of defense-

related genes accumulate as early as 24 hai in Sumai 3 susceptible mutants. A slow defense response in Clark may be the reason why we did not find any induced EST at 6 hai. Still, we could not discount the possibility that this may also be due to low log2 ratio correlation between the 6 hai Clark replication slides. The induction of defense-related ESTs in Clark was observed at 36 hai. This correlates with results from Chapter II showing the down-regulation of Ning 7840 transcripts relative to Clark at 36 hai.

The number of significantly differentially expressed ESTs in Ning 7840 is only about 25% compared to the number of fungal-induced ESTs in Clark. Many ESTs were differentially expressed in Clark but not in Ning 7840. During the course of disease infection, many physiological changes occur in plant cells resulting to shifts in steady state mRNA levels of genes (Wan et al, 2002). Some alterations in gene expression are direct resistance or susceptibility responses while others probably occur as an offshoot of plant disease susceptibility and/or activation of plant defense. Only further characterization of the differentially expressed genes will ascertain the role that these genes may play in FHB resistance.

In this study, the utility of using SSH coupled with microarray to identify *F. graminearum*-induced transcripts was demonstrated. A total of 95 fungal-induced ESTs were identified, eight of which were common between Ning 7840 and Clark. Four out of the nine ESTs showed opposite shifts in transcript abundance between Ning 7840 and Clark. These ESTs did not have significant similarity with other genes in GenBank and may be novel genes that play an important role in FHB resistance. Although some ESTs were assigned with putative functions based on sequence similarity to known proteins, no defense-related function can be presumed for other induced transcripts.

Table 4.1. Significantly differentially expressed ESTs in fungal stressed Ning 7840

Name	Putative ID	E-value	Log2 Ratio
Down-regulated at 6h			
G3-305	No homology		-0.855
36R164	sp|P05167|ALEU_HORVU Thiol protease aleurain precursor	9.00 E-32	-0.712
Down-regulated at 36h			
72R299	No homology		-0.961
Down-regulated at 72h			
6F524	sp|P25413|RBL_AEGCR Ribulose bisphosphate carboxylase large chai...	2.00 E-82	-0.87
Up-regulated at 6h			
G8-157	emb|CAA80492.1| beta glucanase [T. aestivum]	4.00 E-26	0.67
6F447	dbj|BAD07729.1| UDP-glucose pyrophosphorylase [O. sativa]	6.00 E-96	0.65
6R256	Ref|np_566418.1| expressed protein [A. thaliana]	4.00 E-69	0.888
6R264	No homology		0.888
6R347	dbj|AK065654.1| O. sativa	1.00 E-13	0.648
C102	ref|NP_175368.2| long-chain-fatty-acid-CoA ligase	1.00 E-56	0.641
G1-159	gb|AAS49905.1| putative proteinase inhibitor-related protein	1.00 E-39	1.226
Up-regulated at 36h			
72R305	No homology		0.68
72R53	No homology		0.617
G3-12			0.624
G3-273	gi|33147060|dbj|BAC79963.1| unknown protein [O. sativa]	6.00 E-07	0.77
Up-regulated at 72h			
G6-222	No homology		0.839
6F88	dbj|BAB87820.1| P450 [T. aestivum]	7.00 E-32	1.02
72R322	pir||T06985 probable phenylalanine ammonia-lyase (EC 4.3.1.5)	2.00 E-34	0.86

Table 4.2. Down-regulated ESTs in Clark after fungal stress

Name*	Putative ID	E-value	Log2 Ratio
Down-regulated at 36h			
36F327	ref|NP_918065.1| putative fatty acid condensing enzyme CUT1	5.00 E-20	-0.692
36R33	sp|P40880|CAHC_HORVU Carbonic anhydrase, chloroplast	9.00 E-42	-1.105
6F326	No homology		-0.6
6F400	gb|AAQ73182.1| plastidic alpha 1,4-glucan phosphorylase [T. aestivum]	1.0 E-101	-0.909
6F425	dbj|BAC83502.1| putative cyclopropane synthase [O. sativa]	7.00 E-50	-1.057
6F46	dbj|BAD10340.1| pfkB type carbohydrate kinase	3.00 E-18	-0.609
6R130	ref|NP_199987.2| peptidase M48 family protein [A. thaliana]	5.00 E-11	-2.336
6R169	ref|NP_916490.1| P0013F10.1 [O. sativa]	2.00 E-07	-1.15
6R180	No homology		-1.717
6R276	gb|aaq02664.1| boron transporter [O. sativa]	2.00 E-34	-1.421
6R347	dbj|AK065654.1| O. sativa (japonica cultivar)	1.00 E-13	-1.341
72F393	No homology		-0.603
72F75	gb|AAK09371.1| sucrose-6F-phosphate phosphohydrolase SPP1 [T. aestivum]	8.00 E-39	-0.695
72R133			-1.837
72R287	No homology		-1.808
72R53	No homology		-1.685
C102	ref|NP_175368.2| long-chain-fatty-acid-CoA ligase	1.00 E-56	-0.726
C597	emb|CAE51321.1| copper chaperone [H. vulgare]	6.00 E-25	-1.079
G1-153	gb|AAL70109.1|putative aldehyde dehydrogenase	3.00 E-09	-0.853
G1-42	No homology		-1.526
G1-86	No homology		-1.397
G2-368	pir|S47037| tonoplast intrinsic protein gamma [H. vulgare]	8.00 E-12	-0.643
G3-12			-1.392
G8-375	gb|AAK49456.1| glutamine-dependent asparagine synthetase 1 [H. vulgare]	1.00 E-75	-0.884
Down-regulated at 72h			
36F63	emb|X98504.1|TAAMY1 T.aestivum mRNA for beta-lase	7.00 E-58	-0.663
36R381			-0.743
6R247	No homology		-0.636
C597	emb|CAE51321.1| copper chaperone [H. vulgare	6.00 E-25	-1.185
G5-256	gb|AAP80628.1| protein disulfide isomerase [T. aestivum]	6.00 E-08	-0.675
G5-267	sp|P30523|GLGS_WHEAT Glucose-1-phosphate adenylyltransferase small subunit	2.00 E-61	-1.127

*ESTs in boldface are differentially expressed at 36 and 72 hai.

Table 4.3. Up-regulated ESTs in Clark at 36h after fungal stress

Name*	Putative ID	E-value	Log2 Ratio
36F40	ref|NP_910644.1| putative vacuolar ATP synthase subunit H [O. sativa]	2.00 E-57	0.889
36F63	emb|X98504.1|TAAMY1 T.aestivum mRNA for beta-amylase	7.00 E-58	0.972
36R135	dbj|BAD08938.1| putative cytochrome P450 71C4 [O. sativa]	1.00 E-15	1.176
36R287	dbj|BAC57375.2|Putative 1,4-beta-d xylanohydrolase	7.00 E-73	0.923
36R311	gb|AAM63110.1|F-box protein atfbl5 [A. thaliana]	2.00 E-53	0.712
36R353	|ref|NP_915149.1| putative calreticulin [O. sativa.	1.00 E-26	1.918
36R370	No homology		0.926
36R381			0.819
6F176	pir|T06790| thaumatin-like protein precursor [T. aestivum]	1.00 E-19	3.316
6F198	gb|AAG43835.1| protein phosphatase type-2C [Z. mays]	2.00 E-57	0.625
6F350	No homology		0.612
6F429	dbj|BAC83192.1| putative serine/threonine kinase receptor precursor	1.00 E-84	0.717
6F88	dbj|BAB87820.1| P450 [T. aestivum]	7.00 E-32	1.416
6R247	No homology		0.883
72R165	No homology		0.909
72R167	No homology		0.895
72R173	No homology		1.042
72R192	No homology		2.661
72R194	No homology		1.609
72R197	dbj|BAD07637.1| putative Cytochrome P450 [O. sativa]	3.00 E-48	1.425
72R198	No homology		2.239
72R219	No homology		1.276
72R252	No homology		1.808
72R255	gb|AAO45878.1| MADS6 [L. perenne]	5.00 E-50	0.611
72R289	No homology		1.84
72R292	ref|NP_914903.1| putative high-affinity potassium transporter [O. sativa]	4.00 E-60	1.105
72R299	No homology		1.613
72R305	No homology		2.44
72R321	gb|AAP50920.1| putative 3-beta hydroxysteroid dehydrogenase/isomerase	2.00 E-17	1.196
72R47	No homology		1.429
72R54	No homology		1.523
G1-271	No homology		0.806
G1-5	No homology		0.611
G1-81	emb|CAC21228.1| protein disulfide isomerase [T. aestivum]	6.00 E-06	0.73
G1-87	No homology		2.74
G2-247	pir||T04379 probable potassium transport protein	2.00 E-34	0.657
G2-299	gb|AAD28730.1| chitinase II precursor [T. aestivum]	5.00 E-06	2.216
G3-6			0.613
G5-17	dbj|BAC84842.1| PR-1 type pathogenesis-related protein PR-1a [O… sativa]	9.00 E-22	1.689
G5-205	No homology		1.688
G5-211	ref|NP_915149.1| putative calreticulin [O. sativa (japonica cultivar)]	2.00 E-20	1.604
G5-256	gb|AAP80628.1| protein disulfide isomerase [T. aestivum]	6.00 E-08	0.939
G5-313	gb|AAL27005.1| pathogen-related protein [O. sativa]	3.00 E-69	0.701
G5-352	No homology		0.623
G5-90	gb|AAN62909.1| putative glutamate decarboxylase 1 [T. aestivum]	4.00 E-31	0.645

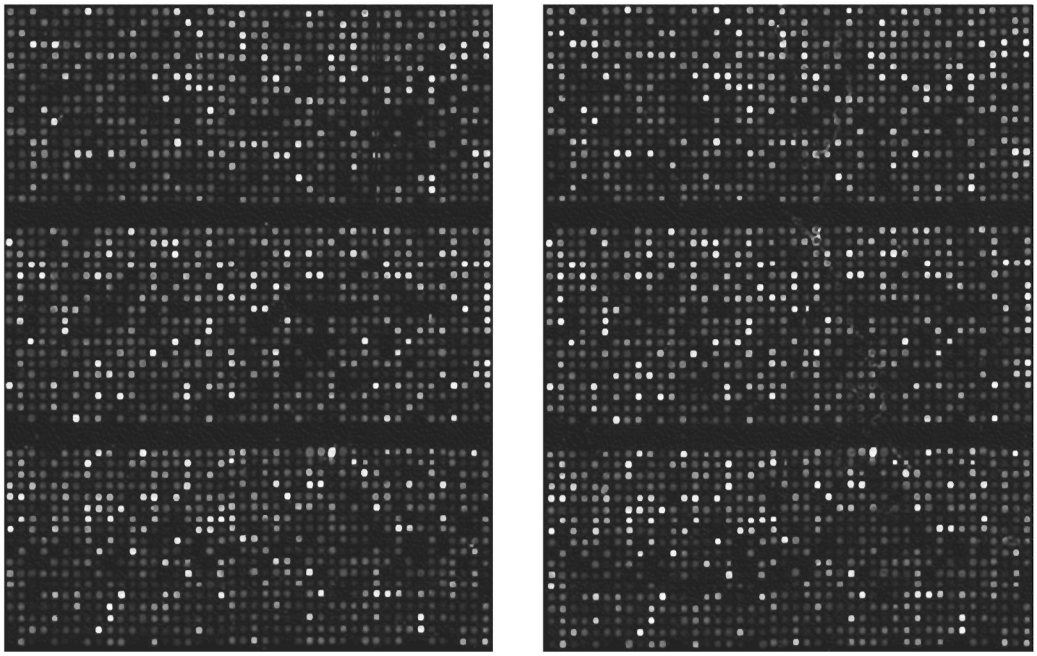
G6-222	No homology	0.699
G6-26	No homology	0.639

*ESTs in boldface are also differentially expressed at 72 hai

Table 4.4. Up-regulated ESTs in Clark at 72h after fungal stress

Name*	Putative ID	E-value	Log2 Ratio
36R135	dbj|BAD08938.1| putative cytochrome P450 71C4 [Oryza sativa]	1.00E-15	1.704
36R278	ref|NP_567724.1|Fibrillarin 2 (fib2) [A. Thaliana]	8.00E-33	1.154
36R353	|ref|NP_915149.1| putative calreticulin [O. sativa]	1.00E-26	0.603
36R40	ref|NP_910644.1| putative vacuolar ATP synthase subunit H [O… sativa]	2.00E-57	1.057
6F429	dbj|BAC83192.1| putative serine/threonine kinase receptor precursor	1.00E-84	0.66
6F88	dbj|BAB87820.1| P450 [T. aestivum]	7.00E-32	1.87
72R162	gb|AAO00709.1|Putative gibberellin 20-oxidase [O. sativa]	2.00E-23	3.278
72R165	No homology		1.683
72R167	No homology		1.042
72R173	No homology		2.493
72R177	ref|NP_922401.1| putative gibberellin oxidase [O. sativa]	2.00E-56	2.905
72R192	No homology		1.893
72R194	No homology		1.618
72R197	dbj|BAD07637.1| putative Cytochrome P450 [O. sativa]	3.00E-48	2.625
72R198	No homology		2.214
72R219	No homology		1.503
72R226	pir||T06985 probable phenylalanine ammonia-lyase (EC 4.3.1.5)	2.00E-34	1.655
72R252	No homology		1.929
72R275	No homology		3.355
72R292	ref|NP_914903.1| putative high-affinity potassium transporter [O. sativa]	4.00E-60	2.782
72R305	No homology		2.179
72R312	No homology		1.36
72R47	No homology		1.047
72R54	No homology		1.549
C102	ref|NP_175368.2| long-chain-fatty-acid--CoA ligase	1.00E-56	0.718
C124	No homology		3.617
C58	dbj|BAD05751.1| putative Aconitate hydratase [O. sativa]	3.00E-95	0.713
C628	emb|CAD59574.1| PDR-like ABC transporter [O. sativa]	3.00E-74	2.133
G1-159	gb|AAS49905.1| putative proteinase inhibitor-related protein	3.00E-39	2.494
G1-87	No homology		1.25
G2-247	pir||T04379 probable potassium transport protein	2.00E-34	0.663
G5-17	dbj|BAC84842.1| PR-1 type pathogenesis-related protein PR-1a [… sativa]	9.00E-22	1.31
G5-211	ref|NP_915149.1| putative calreticulin [O. sativa]	2.00E-20	0.958
G6-222	No homology		1.14
G6-273	dbj|BAA28772.1| alternative oxidase [O. sativa]	7.00E-10	3.241
G6-290	No homology		0.786

*ESTs in boldface are also differentially expressed at 36 hai



A B

Figure 4.1. A pseudocolor image of a portion of a slide hybridized with cDNAs from Ning and Clark sampled at 36 hai with the *F. graminearum*. (A) Fungal- vs. mock-inoculated Ning 7840 and (B) Fungal- vs. mock-inoculated Clark. In both slides, fungal- and mock-inoculated plants were labeled with cy3 (green dye) and cy5 (red dye), respectively. Up-regulated ESTs in the fungal stressed plant are shown as green spots, down-regulated as red, and yellow spots correspond to non-differentially expressed ESTs between the fungal- and corresponding mock-inoculated plant.

CHAPTER V

CONCLUSION

In this study, the utility of using SSH coupled with microarray to identify *F. graminearum*-induced transcripts was demonstrated. A total of ca. 290 significantly differentially expressed ESTs were identified after imposition of fungal stress. The identification of a large number of differentially expressed ESTs suggests that plant defense in response to FHB infection is complex and involves a regulatory network of genes involved in transcription, metabolism, energy generation, protein modification, cell rescue/defense as well as genes of still unknown function. *F. graminearum* appears to have an antagonistic effect on photosynthesis since up-regulation of defense-related ESTs in FHB resistant genotypes (relative to susceptible control) coincided with down-regulation of photosynthesis-related ESTs.

In the resistant genotype, up-regulation of ESTs involved in plant defense and synthesis of signaling compounds occurred as early as 3 hai. These imply that resistance response to FHB occurs before germination of *F. graminearum* and that the plant has a way of sensing the presence of the pathogen. On the other hand, up-regulation of defense- and signal transduction-related ESTs in Clark were observed at 36 hai and coincided with penetration of floral tissues by the pathogen. The results clearly indicate a slower defense response in FHB susceptible compared to resistant genotypes.

A proposed FHB resistance mechanism is shown in Figure 5.1. *F. graminearum* induced the up-regulation of caffeic acid O-methyltransferase (COMT), lipoxygenase (LOX) and UDP-glucose pyrophosphorylase 3 to 6 hai. COMT is a key lignin biosynthetic enzyme and lignification of cell walls provides a stronger barrier against fungal penetration. LOX is probably involved in jasmonic acid (JA)-induced lipid peroxidation of plant cell walls. On the other hand, UDP-glucose pyrophosphorylase

catalyzes the conversion of glucose-1-phosphate to UDP-glucose, a primary metabolite for carbohydrate metabolism. UDP-glucose is used in the production of cellulose and callose (Gibeaut, 2000) which can reinforce cell walls to prevent or reduce fungal penetration. Induction of these ESTs imply that modification of plant cell walls may be a key initial defense response against *F. graminearum.* Transcripts with homology to enoyl CoA hydratase/isomerase were also up-regulated at 3 hai. Enoyl CoA hydratase/ isomerase is an enzyme involved in fatty acid oxidation. LOX and fatty acid oxidation enzymes are involved in the synthesis of JA, the signaling compound involved in most necrotrophic fungi-plant interactions. SGT1 is essential for the activation of some resistance genes or defense signaling (Austin et al. 2002; Glazebrook 2001; Feys and Parker 2000). This gene is a positive regulator of the SCF E3 ubiquitin ligase complex. Interaction of SGT1 with SCF facilitates protein ubiquitilation. An EST putatively identified as SGT1 was up-regulated in the resistant genotype. The up-regulation of SGT1 probably leads to degradation of a negative regulator of the FHB disease resistance pathway so that JA-mediated signaling can proceed and trigger FHB resistance.

In this study, data from real time PCR correlated well with microarray data, therefore real-time PCR is a useful means to validate microarray results.

Resistance to most necrotrophic fungi do not follow gene for gene resistance. One major QTL in 3BS chromosome arm plus a few other minor genes located in other chromosomes governs FHB resistance in Ning 7840. This study identified ESTs that are differentially expressed as a response to *F. graminearum* stress. Although ESTs that had similarity to known genes were identified, the identity or function of the majority is still unknown. Physical and molecular mapping of these ESTs may provide important

information on location of these genes and relationship between these genes and the 3BS major QTL. This may help to further understand the molecular basis of FHB resistance and lay a solid ground for cloning of FHB resistance genes.

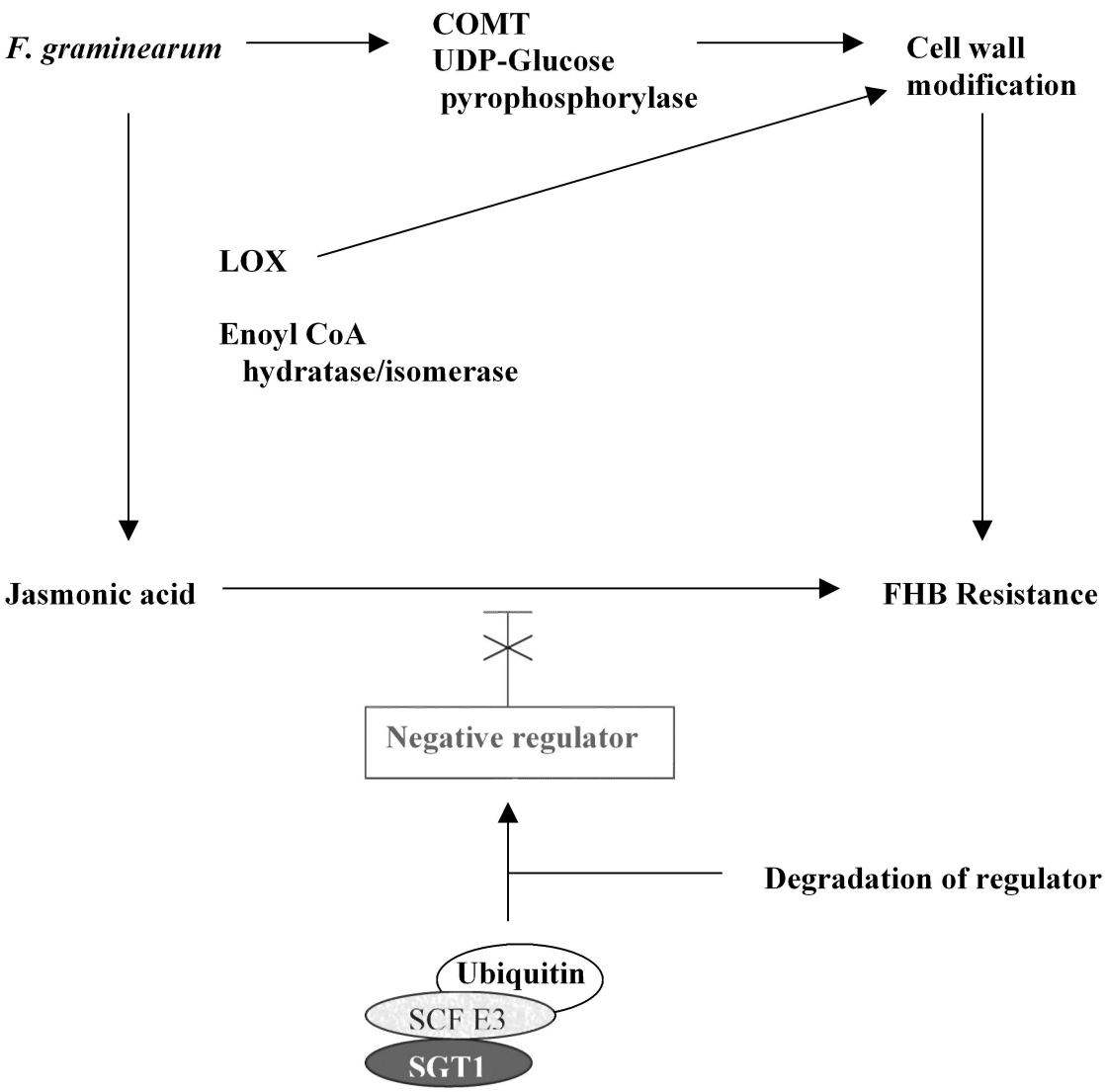


Figure 5.1. Proposed tentative FHB resistance mechanism

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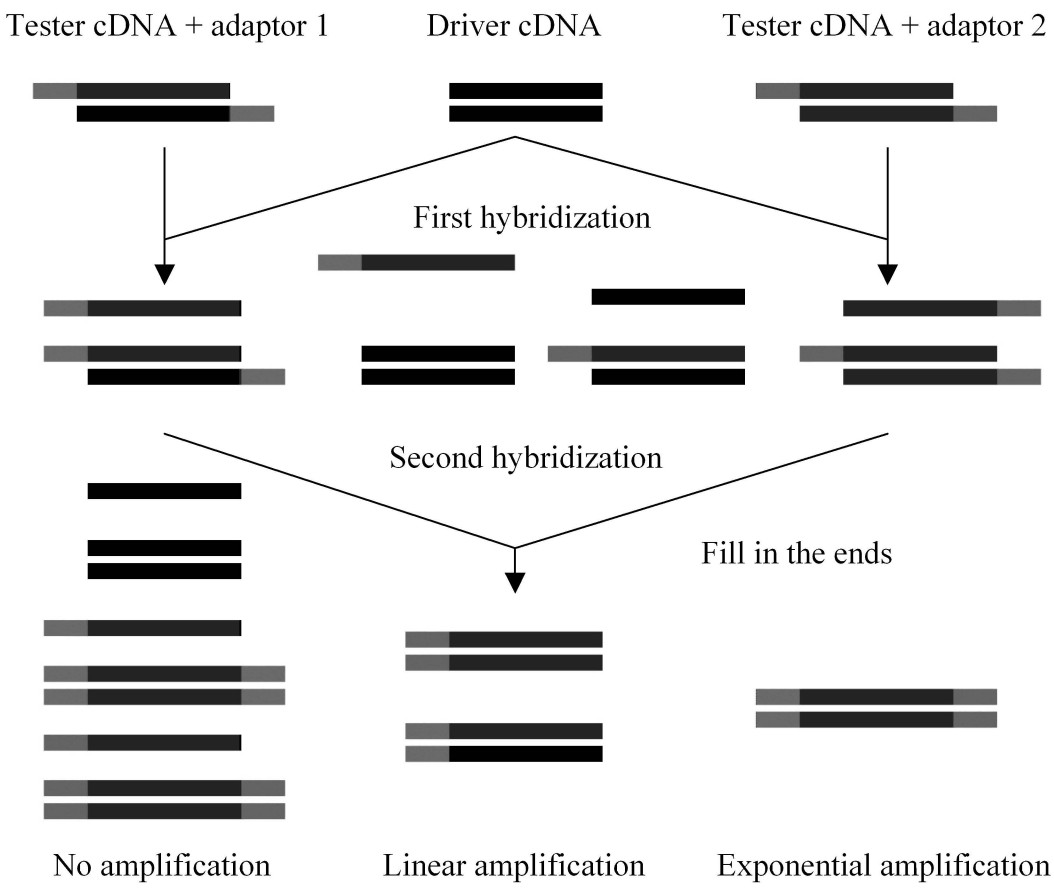
Appendix A
Schematic Diagram of PCR-Select cDNA Subtraction



Appendix B

Log2 ratio of significantly differentially expressed ESTs between Ning 7840 and Clark

Name	0 hai	3 hai	6 hai	12 hai	24 hai	36 hai	48 hai	72 hai	Putative ID
36F1	-0.613	-0.678	0.049	0.07	-0.174	0.197	0.029	-0.165	gb|AB020954.1| ribulose-1,5-bisphosphate carb small sub
36F150	-0.239	-0.582	-0.539	-1.128	-0.378	0.263	-0.794	-1.373	dbj|BAB01742.1| unnamed protein product [Arabidopsis thaliana]
36F157	0.337	0.557	1.15	0.818	0.385	0.699	0.58	0.843	No homology
36F281	-0.779	-0.606	-0.387	-0.254	0.215	-0.022	0.8	0.644	dbj|BAC83538.1| putative cytoplasmic ribosomal protein L18 [Oryz...
36F314	0.57	0.825		1.144	1.401	1.559	1.377	1.659	
36F59	1.426	0.778	-0.674	1.715	1.743	0.501	0.72		dbj|BAC92507.1| putative fatty acyl coA reductase [Oryza sativa]
36F63	-0.077	0.085	-1.08	-1.122	-0.38	-0.539	0.197	0.182	emb|X98504.1|TAAMY1 T.aestivum mRNA for beta-amylase
36F90	1.039	0.938	1.157	3.139	2.292	0.567	0.805	1.244	emb|CAD41786.2| OSJNBa0008M17.1 [Oryza sativa]
36R1	-2.048	-1.056	-4.095	-2.89	-2.823	-0.705		-1.663	hypothetical protein
36R110	-0.553	-0.594	-0.551	-1.009	-0.895	-0.478	-0.365	-0.466	ref|NP_912998.1| unnamed protein product [Oryza sativa]
36R135	1.987	1.346	0.427	-0.421	-0.746	-1.548	-2.306	-1.53	dbj|BAD08938.1| putative cytochrome P450 71C4 [Oryza sativa]
36R170	-2.66	-1.754				-1.101		-1.858	No homology
36R27	-0.504	-0.604	-0.154	-0.34	-0.227	0.033	-0.119	-0.08	gb|AAO45878.1| MADS6 [Lolium perenne]
36R33	-0.719	-0.673	0.41	0.352	0.767	0.778	0.505	0.08	sp|P40880|CAHC_HORVU Carbonic anhydrase, chloroplast
36R349	0.095	0.6	0.481		0.261	0.198	-0.173	-0.597	sp|Q8GSM2| Lipoxygenase 2.3, chloroplast
36R353	0.171	-0.103	-0.446	0.225	-0.79	-1.578	-1.339	-1.151	ref|NP_915149.1| putative calreticulin [Oryza sativa]
36R359	-0.446	-0.581	-0.664	-1.147	-0.904	-0.573	-0.4	-0.395	ref|NP_916283.1| putative Regulatory protein NPR1
36R40	0.367	0.602	-0.337	0.322	0.269	0.176	-0.46	-0.243	No homology
36R45	0.221	0.288	0.631	0.971	0.515	0.157	0.428	0.534	
36R78	-0.843	-0.818	-0.566	0.712	1.373	0.458	-0.601	-0.24	No homology
6F1	0.695	0.09			1.316	0.011		0.662	gb|AAO19364.1| hypothetical protein [Oryza sativa

6F166	-0.593	-0.612	-0.229	-0.273	-0.12	0.088	-0.053	-0.25	pir|S53306| MADS box protein MADS1 - rice
6F176	-0.559	-1.046				-3.727	-3.524	-4.4	pir|T06790| thaumatin-like protein precursor
6F19	1.561	0.498		0.159		0.452	0.517	1.345	ref|NP_921964.1| putative serine protease [Oryza sativa]
6F202	-0.589	-0.741	0.039	0.056	-0.115	0.005	0.312	0.074	dbj|BAB19810.1|Ribulose-1,5-bisphosphate carboxylase/oxygenase
6F220	2.636	2.675	3.31	3.121	3.468	2.322	2.32	3.136	ref|NP_917762.1| P0501G01.24 [Oryza sativa]
6F222	-0.58	-0.828	-0.453	-0.017	-0.017	-0.111	-0.032	0.131	ref|NP_565093.1| expressed protein [Arabidopsis thaliana]
6F270	1.357	1.156				0.257	0.642	1.356	
6F279	-0.717	-0.747	-0.105	-0.164	-0.069	0.066	0.11	-0.074	dbj|BAC83859.1| putative N-amidino-scyllo-inosamine-4-phosphate
6F326	0.325	0.696	0.85	0.839	0.941	0.719	0.663	0.441	No homology
6F330	-0.545	-0.646	-0.619	0.012	-0.08	-0.239	-0.509	-0.348	ref|NP_920160.1| putative crp1 protein [Oryza sativa]
6F379	-0.345	-0.871	-0.196	-0.853	-0.466	0.095	0.35	-0.089	No homology
6F386	0.068	0.274	0.955	1.692	0.846	0.57	0.603	0.408	sp|P26517|G3PX_HORVU Glyceraldehyde 3-phosphate dehydrogenase
6F389	-1.696	-1.373	-0.67	-0.203			-0.885		No homology
6F420	1.951	1.365				1.265	1.364	1.671	gb|AAS88823.1| unknown protein [Oryza sativa
6F427	0.126	-0.017	0.577	1.582	1.534	0.886	0.508	0.579	emb|CAA25058.1| ribulosebisphosphate carboxylase [Triticum aestivum]
6F474	0.614	0.65	0.725		0.212	0.324	0.025	-0.153	gb|AAK07429.1| beta-glucosidase [Musa acuminata]
6F5	1.428	1.273	0.323	1.79	0.83	0.317	-0.411	0.104	gb|AAG51044.1| kinesin heavy chain, putative
6F524	0.155	-0.528	0.567	0.391	0.304	0.979	-0.248	0.016	sp|P25413|RBL_AEGCR Ribulose bisphosphate carboxylase large chain
6F541	-0.65	-0.664	0.219		-0.046	0.01	-0.337	-0.372	gb|AAS07184.1| putative peroxisomal membrane protein [Oryza sativa]
6F61	-0.134	-0.015	0.908	2.273	0.282	0.044	0.127	0.372	No homology
6F88	1.712	1.111	0.492	-1.104	-1.423	-1.794	-2.344	-1.975	dbj|BAB87820.1| P450 [Triticum aestivum]
6F89	2.735	2.908	3.128	3.183	3.439	2.947	2.293	3.263	No homology
6R130	-1.21	-1.711	-0.583	0.219	1.341	0.394	-0.497	-0.24	ref|NP_199987.2| peptidase M48 family protein [Arabidopsis thaliana]
6R132	-0.407	0.615	0.17	-0.44	-0.274	-0.663	-0.488	-0.609	No homology
6R169	0.919	1.22	1.545	1.91	2.631	2.125	0.946	1.902	ref|NP_916490.1| P0013F10.1 [Oryza sativa]
6R177	-0.532	-0.69	-0.327	-0.295	-0.165	-0.226	-0.023	-0.046	ref|NP_175137.1| chlorophyll A-B binding protein, putative

6R224	-1.068	-0.537		-1.289	-1.119	-0.112	-0.821	-0.776	No homology
6R247	-0.218	0.104	-1.204	-1.256	-0.345	-0.53	0.218	0.16	No homology
6R264	-0.694	-0.451	1.39				-0.222		No homology
6R279	0.299	0.016	0.66	1.191	0.119	-0.184	-0.324	0.094	No homology
6R286	-0.851	-0.534	0.917	1.442		-0.1	-0.118		pir|T06550| pollen allergen homolog - wheat
6R347	-1.079	-1.398	-0.426	0.215	-0.175	0.449	-0.416	-0.578	dbj|AK065654.1| Oryza sativa (japonica cultivar)
6R352	-0.57	-0.519	0.204	0.335	0.633	0.111	0.451	-0.121	gb|B23703| ribulose-bisphosphate carboxylase activase
6R517	-0.503	-0.606	0.015	0.109	-0.106	-0.009	-0.133	-0.253	gb|AAM88439.1| putative Rieske Fe-S precursor
6R66	-0.554	-0.573	-0.628	-0.624	-0.692	-1.061	-0.51	-0.54	No homology
6R80	-0.538	-0.889	-0.521	-0.437	-0.343	-0.272	-0.245	-0.39	No homology
6R81	0.55	0.543	0.812	0.76	0.893	0.659	0.712	0.435	ref|NP_916222.1| hypothetical protein
6R91	-0.586	-0.364	0.821	1.407	0.863	0.252	-0.069	-0.011	No homology
6R93	-0.722	-0.623	0.114	0.297	0.28	0.145	0.419	-0.05	pir||B23703 ribulose-bisphosphate carboxylase activase
72F104	-0.651	-0.739	0.11	0.018	-0.018	0.061	0.207	0.057	unknown protein
72F117	0.494	0.457	0.4	0.456	0.909	0.64	0.322	0.36	dbj|BAC83558.1| unknown protein [Oryza sativa (japonica cultivar)
72F122	0.286	0.538	0.745	0.896	0.857	0.6	0.649	0.376	sp|P30523|GLGS_WHEAT Glucose-1-phosphate adenylyltransferase
72F123	-0.138	0.007	-0.66	-1.05	-0.395	-0.734	0.295	0.201	emb|Z11772.1|SCBAMYLM S.cereale mRNA for beta-amylase
72F21	0.381	0.541	0.622	0.617	0.95	0.715	0.6	0.546	No homology
72F237	0.423	0.556	0.459	0.542	0.706	0.604	0.338	0.233	No homology
72F247	0.265		0.579	0.559	0.812	0.653	0.667	0.165	
72F254	0.468	0.666	0.55	0.517	0.942	0.699	0.456	0.592	No homology
72F28	0.56	0.575	0.774	0.615	0.946	0.559	0.692	0.459	No homology
72F289	0.883	0.42	0.701	0.504	0.328	0.254	0.268	0.494	gb|AAD04231.1| PDI-like protein [Zea mays]
72F295	-0.605	-0.717	0.227	-0.246	0.151	0.056	0.029	0.14	ref|NP_914894.1| OSJNBa0052O12.12 [Oryza sativa]
72F323	0.343	0.549	0.673	0.441	0.768	0.509	0.441	0.374	gb|AAO38465.1| putative serine carboxypeptidase I [Oryza sativa]
72F339	0.247	0.647	0.481	0.021	0.513	0.829	0.908	0.516	No homology
72F366	0.463	0.757	0.549	0.401	1.098	0.667	0.468	0.283	No homology
72F374	0.481	0.582	0.82	0.704	0.977	0.565	0.598	0.386	No homology

72F393	0.678	0.597	0.642	0.634	0.893	0.699	0.64	0.322	No homology
72F394	0.691	0.379	0.537	0.589	0.221	0.287	0.339	0.538	No homology
72F399	0.631	0.594	0.574	0.585	0.832	0.628	0.587	0.35	
72F52	0.405	0.486	0.561	0.813	0.858	0.582	0.453	0.399	No homology
72F75	0.509	0.513	0.764	0.658	0.807	0.68	0.619	0.247	gb|AAK09371.1| sucrose-6F-phosphate phosphohydrolase SPP1 [Triticum aestivum]
72F82	0.588	0.672	0.764	0.785	1.143	0.761	0.774	0.196	No homology
72R133	-0.178	-0.424	0.809	0.267	0.626	1.083	0.033	0.851	
72R149	0.075	-0.352	-0.43		-0.48	-0.315	-1.528	-1.69	emb|CAD41042.1| OSJNBa0060P14.1 [Oryza sativa]
72R154	-0.946	-0.801		-0.961	-1.198	-0.699	-0.564		
72R162	0.681	0.427				-0.897	-2.925	-2.369	gb|AAO00709.1|Putative gibberellin 20-oxidase [Oryza sativa]
72R164	0.497	0.459		0.132		-0.588	-2.577	-2.364	ref|NP_922401.1| putative gibberellin oxidase
72R165	1.508	1.179	0.217	-1.379	-1.398	-1.628	-2.436	-1.95	No homology
72R167	0.204	0.105	0.202	0.136	0.01	-0.861	-0.74	-1.389	No homology
72R170	1.151	1.545				-0.006	-0.32	-1.344	emb|CAE03959.2| OSJNBb0085H11.8 [Oryza sativa]
72R173	0.908	0.895	-0.2	-0.112	-1.209	-1.149	-2.31	-2.001	No homology
72R177	0.624	0.15				-0.665	-2.603	-2.379	No homology
72R191	0.22	0.039	0.153	-0.281	-1.549	-1.134	-1.444	-1.7	No homology
72R192	0.375	0.655	0.123	0.524	0.13	-2.09	-0.942	-2.065	No homology
72R194	0.198	0.278	-0.301	0.362	-0.694	-1.954	-1.143	-2.121	No homology
72R197	0.883	0.289			-1.933	-1.547	-2.842	-2.484	dbj|BAD07637.1| putative Cytochrome P450 [Oryza sativa]
72R198	2.136	0.698		-0.414	-1.36	-1.536	-1.38	-2.867	No homology
72R2	1.024	2.049				-0.092		-2.106	emb|X96429.1| Cadinene synthase [Gossypium arboreum]
72R206	0.283	0.31	0.403	0.44	0.65	-0.899	-0.618	-1.217	No homology
72R214	1.023	2.136	0.294			-0.021	-0.392	-1.968	emb|CAD40467.2| OSJNBa0067G20.21 [Oryza sativa]
72R219	0.257	0.238	0.186	0.288	-0.02	-1.412	-0.943	-1.987	No homology
72R252	0.26	0.277	0.246	0.419	-0.111	-1.581	-1.342	-2.346	No homology
72R275	0.299	0.332	-0.188	-0.241	-0.725	-0.507	-2.151	-1.996	No homology
72R282	-0.747	-0.747	-0.555	-0.71	-0.988	-0.927	-0.632	-0.583	No homology
72R287	-0.122	-0.287	0.881	0.458	0.63	1.106	0.09	0.704	No homology
72R289	0.275	0.227	-0.238	0.166	-0.505	-1.534	-1.225	-2.063	No homology

72R292	1.838	0.749		-0.025		-1.497	-1.676	-1.591	ref|NP_914903.1| putative high-affinity potassium transporter
72R299	0.108		-0.055	-1.189	-1.485	-1.316	-0.087	0.086	No homology
72R305	1.891	0.954	-0.101	-0.652	-1.116	-1.328	-1.43	-2.904	No homology
72R312	0.148		-0.036			-1.035	-1.258	-1.674	No homology
72R320	0.448	0.403	-0.292	-0.1	-0.603	-1.33	-1.139	-1.656	gb|AAK55326.1| thaumatin-like protein TLP8 [Hordeum vulgare]
72R321	0.409	0.3	-0.181	0.358	-1.993	-1.56	-2.243	-2.321	gb|AAP50920.1| putative 3-beta hydroxysteroid dehydrogenase/isomerase
72R47	0.453	0.406		0.779	-0.325	-1.12	-1.475	-1.634	No homology
72R53	-0.145	-0.344	0.87	0.641	0.602	1.133	0.215	0.906	No homology
72R54	0.479	0.669	-0.559	-0.146	-0.436	-1.444	-1.117	-1.746	No homology
72R79	0.091	0.423	0.274	1.319	0.785	0.433	-0.585	-0.304	No homology
72R8	-0.117	-0.091	-0.741	-0.812	-0.45	-0.342	-0.027	-0.216	No homology
72R80	0.23	0.096			-0.101		-1.296	-1.526	ref|NP_916475.1| putative MRP-like ABC transporter [Oryza sativa]
A769	0.529	0.803	-0.023	0.149	0.43	0.15	0.286	0.349	gb|AAO72664.1| wheat adenosylhomocysteinase-like protein [Oryza sativa]
A779	0.645	1.035	0.466	0.743	0.188	0.623	-0.144	0.763	
C124	0.748	0.166		0.904	0.173	0.21	-2.273	-2.394	No homology
C150	1.464	1.978	-1.004	-1.734	-1.552	-0.629	-1.032	-1.828	pir||T06181 subtilisin-chymotrypsin inhibitor 2 - barley
C597	-0.698	-0.445	-0.593	-0.356	0.733	1.025	0.254	0.726	emb|CAE51321.1| copper chaperone [Hordeum vulgare]
C62	0.276							-2.27	gb|AAL60592.1| cytochrome P450 monooxygenase CYP72A26 [Zea mays]
C69	0.294		0.044	0.53		-1.136	-1.169	-2.108	emb|CAD59574.1| PDR-like ABC transporter [Oryza sativa]
C834	0.217	0.748	-0.319	-0.091	0.091	0.015	0.209	0.012	gb|AAL73979.1| methionine synthase protein
C839	0.182	0.649	-0.305	-0.138	0.239	0.113	0.305	0.03	No homology
G1-159	1.544	1.968	0.151	-0.914	-1.166	-0.165	-1.729	-1.765	gb|AAS49905.1| putative proteinase inhibitor-related protein
G1-177	-0.739	-0.678	-0.289	-0.196	-0.462	-0.707	-0.362	-0.508	emb|CAD23409.1| putative MADS-domain transcription factor
G1-179	0.39	0.818	0.011	-0.363	-0.093	0.007	-0.03	-1.063	No homology
G1-295	0.202	0.967	0.52	0.535	0.592	0.196	0.236	-0.051	ref|NP_914529.1| unnamed protein product [Oryza sativa]

G1-369	-0.065	-0.07	0.461	0.905	0.448	0.328	0.293	-0.037	No homology
G1-76	-0.042	0.065	0.455	1.005	0.179	0.364	0.084	0.076	No homology
G1-86	-0.157	-0.307	0.776	0.61	0.776	0.97	0.12	1.025	No homology
G1-87	1.541	1.014	-0.015	-0.058	-0.877	-2.136	-1.464	-1.643	
G2-119	-0.882	-0.154			0.142		0.116		No homology
G2-284	-1.733	-1.707	-1.656	-1.731	-1.02	-1.296	-1.199	-1.589	No homology
G2-287	-0.688	-0.717	-0.268	-0.781	-0.432	-0.525	-0.315	-0.546	dbj|BAB82503.1| CIG1 [Nicotiana tabacum]
G2-299	1.28	0.987			-0.897	-2.09	-1.836	-2.155	gb|AAD28730.1| chitinase II precursor [Triticum aestivum]
G2-360	0.227	0.22	0.762	1.189	0.706	0.289	0.017	-0.014	sp|P37707|B2_DAUCA B2 PROTEIN
G2-374	0.261	0.62	0.238	0.098	0.006	-0.226	-0.061	0.117	
G3-141	0.984	1.365	-0.541	-0.721	-0.934	-0.206	-1.238	-1.687	
G3-156	0.26	0.25	0.739	1.26	0.592	0.288	-0.023	0.012	
G3-239	-0.001	0.155	0.538	1.156	0.728	0.496	0.331	0.134	
G3-260	-0.037	-0.011	0.463	1.171	0.361	0.306	0.341	0.058	
G3-273	-0.201	-0.645	0.423	0.255	-0.066	-0.071	-0.281	-0.953	dbj|BAC79963.1| unknown protein [Oryza sativa]
G3-298	-0.037	0.337	0.617	0.339	0.6	0.298	0.394	0.164	
G3-329	-0.507	-0.733	0.878			-0.113	-0.231	-0.274	
G3-334	0.768	0.956	-0.104	-0.347	-0.69	-0.296	-0.921	-1.775	
G3-339	0.276	0.641	-0.197	0.391	0.358	0.231	-0.211	-0.22	
G3-350	0.919	0.867	0.364	0.319	0.718	0.511	0.554	0.759	ref|NP_910169.1| putative disulfide-isomerase precursor
G3-351	0.157	0.199	0.703	1.447	0.57	0.545	0.545	0.346	
G3-357	-0.639	-0.451	-0.153	-0.696	-0.241	-0.138	0.375	-0.173	
G4-101	-0.978	-0.18	-0.315	-0.35	0.093		-0.069	-0.135	
G4-266	-1.541	-1.615	-1.649	-1.691	-1.105	-1.124	-1.227	-1.378	No homology
G4-269	-0.567	-0.669	-0.376	-0.934	-0.465	-0.436	-0.363	-0.401	ref|NP_922646.1| putative proline oxidase [Oryza sativa]
G4-29	0.366	0.207	0.807	0.767	0.963	0.791	0.556	0.291	
G5-13	-0.448	-0.185	-0.337	-0.701	-0.24	0.016	0.212	0.265	No homology
G5-145	0.479	0.713	0.343	0.388	0.075	0.473	0.427	0.452	No homology
G5-16	0.759	1.04	0.709	0.93	0.222	0.519	0.452	1.07	
G5-17	1.154	0.904	-0.105	0.572	-0.359	-1.808	-0.803	-1.911	dbj|BAC84842.1| PR-1 type pathogenesis-related protein PR-1a
G5-185	0.762	1.203	0.476	0.827	0.301	0.764	0.717	1.113	No homology

G5-196	0.161	0.337	0.53	1.007	0.595	0.231	0.397	0.496	
G5-205	0.679	0.168	-0.429	0.021	-0.633	-2.027	-1.098	-1.505	No homology
G5-264	0.137	-0.56	0.151	0.288	0.407	-0.109	-0.295	-1.139	gb|AAK25768.1| auxin-repressed protein like-protein
G5-278	0.964	1.168	0.694	0.914	0.327	0.475	0.585	1.218	No homology
G5-297	0.615	0.56	0.397	-0.055	0.283	0.182	0.19	0.257	ref|NP_172142.2| enoyl-CoA hydratase/isomerase family protein
G5-299	1.002	0.352	0.27	0.582	0.193	0.387	0.181	0.458	gb|AAP50932.1| putative trypanothione-dependent peroxidase
G5-31	0.589	1.023	0.518	0.744	0.298	0.515	0.394	0.926	No homology
G5-313	0.06	0.602	0.127	-0.437	-0.658	-0.526	-1.104	-1.155	gb|AAL27005.1| pathogen-related protein [Oryza sativa]
G5-345	0.063	-0.059	0.043	0.65	0.146	-0.095	-0.154	-0.111	
G5-364	0.255	0.13	0.476	1.179	0.651	0.275	0.229	0.194	
G5-37	0.235	0.221	0.638	0.712	0.612	0.344	-0.023	-0.071	dbj|BAB03377.1| unnamed protein product [Oryza sativa]
G5-370	-0.029	-0.142	0.163	1.225	0.164	-0.052	-0.164	-0.098	ref|NP_200670.1| zinc finger (CCCH-type) family protein
G5-95	0.355	0.765	-0.164	0.842	0.731	1.468	0.631	1.101	emb|CAE02970.2| OSJNBb0079B02.2 [Oryza sativa]
G6-108	0.691	1.083	0.669	0.876	0.107	0.574	0.604	0.705	No homology
G6-111	-0.318	-0.203	-0.078	0.342	0.382	0.889	0.588	0.828	
G6-133	0.56	0.993	0.446	0.91	0.04	0.55	0.446	0.84	No homology
G6-157	0.313	0.768	0.595	0.052	0.173	0.045	0.039	0.152	
G6-18	1.159	0.473	1.752	0.665	0.987	0.553		0.996	No homology
G6-198	0.795	1.154	0.786	0.841	0.246	0.506	0.673	1.445	No homology
G6-222	0.588	0.619	0.177	-0.097	-0.306	-0.351	-0.748	-0.67	No homology
G6-236	0.329	0.61	-0.174	0.233	0.432	0.616	0.373	0.249	gb|AAP23942.1| caffeic acid O-methyltransferase
G6-253	0.279	0.473	0.671	1.09	0.81	0.336	0.391	0.623	No homology
G6-271	0.169	0.334	0.623	1.025	0.641	0.26	0.524	0.473	No homology
G6-273	0.464	0.346				-0.166	-2.58	-1.781	dbj|BAA28772.1| alternative oxidase [Oryza sativa]
G6-290	0.24	-0.14	-0.336	0.119	-0.852	-1.619	-1.312	-0.939	No homology
G6-291	0.673	0.603	0.563	0.199	0.212	0.158	0.098	0.448	
G6-296	-0.1	0.323	-0.099	0.823	0.633	1.129	0.558	0.749	No homology
G6-33	0.327	0.738	-0.372	0.492	0.22	0.254	-0.403	-0.341	ref|NP_914769.1| putative phospho-2-dehydro-3-deoxyheptonate aldolase
G6-376	0.671	0.865	0.579	1.136	0.241	0.691	0.702	0.847	

G6-55	-0.196	-0.701	0.432	0.258	0.14	0.448	-0.148	-0.192	No homology
G6-78	0.287	0.666	0.168	-0.166	-0.699	-0.456	-1.378	-1.503	No homology
G8-106	0.91	0.869	0.672	0.857	0.199	0.631	0.431	0.836	No homology
G8-157	0.012	-0.486	0.467	0.087	-0.16	-0.692	-0.029	-1.87	emb|CAA80492.1| beta glucanase [Triticum aestivum]
G8-178	-0.223	-0.119	-0.456	-0.734	-0.267	-0.142	0.349	0.099	ref|NP_912437.1| Putative transporter [Oryza sativa]
G8-275	0.186	0.605	-0.278	-0.187	0.215	0.052	0.201	-0.12	emb|CAA89019.1| cobalamine-independent methionine synthase
G8-28	0.289	0.624	-0.387	-0.478	0.16	0.079	0.195	0.114	gb|AAM28274.1| PFE18 protein [Ananas comosus]
G8-34	0.65	0.196	0.941	0.667	0.241	0.04	-1.78	-2.414	No homology
G8-375	0.494	1.155	1.689	-0.512	-0.135	0.491	-0.751	-0.42	gb|AAK49456.1| glutamine-dependent asparagine synthetase 1 [Horduem vulgare]

VITA

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