SESSION 1:

GENE DISCOVERY AND ENGINEERING RESISTANCE

Co-Chairpersons: Steve Scofield and Jyoti Shah

CHARACTERIZATION OF WHEAT CYTOCHROME P450S UP-REGULATED AS AN EARLY RESPONSE TO THE *FUSARIUM* MYCOTOXIN DEOXYNIVALENOL

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ABSTRACT

Using transcript-profiling studies, we identified two cytochrome P450 (CYP) transcripts (CYP1724 and CYP840) up-regulated in wheat spikelets as an early response to DON treatment (5 mg ml^{-1}; 4 h post-treatment). This toxin-induced accumulation was found to be associated with the DON tolerance of cultivar CM82036 contributed by the quantitative trait locus (QTL) *Fhb1* (chromosome 3BS). Using real time RT-PCR analysis, the temporal accumulation (1 – 4 h) of these transcripts in roots of DON-treated (20 mg ml^{-1}) wheat seedlings (cultivar CM82036) was determined. In roots of DON treated seedlings, the CYPs were induced within 1 h and their levels reached a maximum at 3.5 h post-DON treatment. In seedlings of cultivar CM82036, both the CYP transcripts were induced 1.7 fold in salicylic acid-treated roots, while only CYP840 transcripts were 3.2 times more abundant in jasmonic acid-treated roots at 4 h post-treatment as compared to control roots ($P < 0.001$). The CYPs expression in coleoptiles of seedlings of cultivar CM82036 (DON resistant) and cultivar Remus (DON susceptible) whose roots were treated with 20 μg ml^{-1} of DON for 24 h in light and dark conditions was analyzed. Although no detectable levels of CYP 840 transcripts were found in coleoptiles of both cultivars, CYP1724 transcripts were induced in coleoptiles both by DON and light in both genotypes. While CYP1724 expression levels did not differ among genotypes under dark, its expression levels were 2.37 times higher in coleoptiles of DON-treated seedlings of cultivar CM82036 than of cultivar Remus when incubated in light, suggesting a light dependant DON tolerance in wheat. Further characterization of the CYPs is being carried out using heterologous expression systems.

IDENTIFICATION OF A DIRECT ROLE FOR MITOCHONDRIA IN TRICHOTHECENE RESISTANCE

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ABSTRACT

Trichothecenes produced by various species of *Fusarium* are increasingly contaminating cereal crops worldwide. *Fusarium graminearum* causes Fusarium head blight (FHB) in both wheat and barley resulting in reduced plant yield and contamination of the grains with trichothecenes, in particular DON. Improving FHB resistance, hence, remains a high priority in wheat and barley breeding programs throughout the world. Identifying the molecular mechanisms underlying trichothecene toxicity is therefore vital to understanding *Fusarium* pathology and engineering FHB resistance.

We have previously shown that mitochondria are critical for trichothecin (Tcin) toxicity in yeast. Sensitivity to Tcin increased when yeast cells were grown in non-fermentable media, which requires functional mitochondria, while cells devoid of mitochondria (ρ^0) showed increased resistance to Tcin. Over 60% of gene deletions that conferred resistance to Tcin were associated with mitochondrial function in our genome wide screening of the yeast deletion library. Moreover, mitochondrial translation was shown to be inhibited by Tcin in the wild type but not in the resistant mutants. To determine if Tcin has a direct effect on mitochondria, we examined translation in isolated yeast mitochondria treated with Tcin. Furthermore, we employed flow cytometry to assess functionality of the yeast mitochondria when treated with trichothecenes.

A 60% inhibition in translation was observed in isolated yeast mitochondria treated for 10min with 4μM Tcin, solubilized in 50% ethanol, when compared to mitochondria treated with 50% ethanol. This inhibition increased to 78% at 8μM Tcin suggesting a direct inhibition of mitochondrial translation by Tcin. Flow cytometric analyses of Tcin-treated yeast cells stained for mitochondrial membrane potential, ROS generation and cell death also suggest a role for mitochondria in Tcin-induced cell death. Peak shifts in the median fluorescence intensities of Tcin-treated cells indicate that Tcin triggers ROS generation resulting in hyperpolarization of the mitochondrial membrane which eventually leads to cell death.

SEQUENCING AND PRELIMINARY ANALYSIS OF CHROMOSOME 2H BIN 10 PREDICTED GENES

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OBJECTIVES

Map and sequence barley chromosome 2H bin 10 region in order to identify candidate genes for Fusarium head blight resistance.

INTRODUCTION

Fusarium Head Blight (FHB) is a serious disease of barley and wheat that has been difficult to control due to lack of available Mendelian resistance genes. Nevertheless, numerous quantitative trait loci (QTL) conferring variable degrees of resistance have been identified. One strong, recurring FHB resistance QTL was identified on chromosome 2H bin 10 (Canci et al., 2004; Dahleen et al., 2003; de la Pena et al., 1999; Hori et al., 2005; Horsley et al., 2006; Ma et al., 2000; Mesfin et al., 2003). The chromosome 2H bin 10 QTL has been ascribed to the 2-rowed head type present in one of the resistant parents CIho 4196 (Zhu et al., 1999). This idea has been discredited by the development of a 6-rowed CIho 4196 mutant that has consistently shown FHB resistance comparable to the parent CIho 4196 (Boyd et al., 2008) and selection of 6-rowed recombinants from crosses of CIho 4196 by susceptible cultivars that have maintained FHB resistance (Kleinhofs et al., unpublished; Horsley et al., unpublished). The chromosome 2H bin 10 region from MWG865 to MWG503 has been the focus of our research to develop a saturated genetic and physical map. Recently we have narrowed this region from BF265762A to ctg15522. This region covers approximately 3.5 cM and is saturated with 15 BAC contigs containing a minimal tiling path of 31 BAC clones. Sequencing and preliminary analysis of these BAC clones is reported.

MATERIALS AND METHODS

BAC DNA isolation and sequencing - BAC DNA was isolated using the NucleoBond BAC 100 kit (Clonetech, Mountain View, CA, USA) per manufacturers instructions. The quality and quantity of the individual BAC DNA was evaluated by agarose gel separation and using nanodrop technology. Individiual BAC clones were quantified and adjusted to ca.1ug/ul and pooled for construction of the libraries. The 454 libraries were prepared by fragmenting ca. 10 ug pooled BAC plasmid DNA using a nebulizer. The fragmented DNA was run on a 1% agarose gel and size selected for fragments of 400-600 bp by isolating the appropriate band from the gel. The gel slice was extracted using the Qiagen (Valencia, CA, USA) gel elution kit (to isolate and purify the fragmented DNA. The purified fragments were evaluated for size and quantity using a BioAnalyzer genechip. Fragmented DNA was ligated to the provided adaptors and purified using oligotex beads. An additional size and quantity verification was run using a BioAnalyzer genechip. Libraries were evaluated for concentration by titration according to the manufacturer's protocols and sequenced on three regions of a four-region gasket. Sequencing was performed using the Genome Sequencer FLX titanium series protocol (Roche 454 Life Sciences).

Sequence analysis - Sequences were assembled by the Genome Sequencer FLX system software. Contigs from the analysis were screened using the BLASTx function at the NCBI website to eliminate *E. coli* contamination. The remaining contigs were analyzed in Softberry FGENESH (linux1.softberry.com) for gene prediction. Predicted genes were then screened using the BLASTx function to sort out putative

retro elements and to assign putative function to other genes.

Southern probe development and mapping to BAC clones - Predicted genes were screened against the *Hordeum vulgare* database of NCBI using the BLASTn function limited to "EST others" to find ESTs in our library. Those ESTs were hybridized to BAC filters containing the BACs sequenced in the appropriate region in order to connect the sequenced contig with the correct BAC.

Primer development and re-mapping to individual BAC clones - When no EST was available from a contig, primers were designed and amplified using a touchdown PCR protocol performed on the twelve BACs of the region to localize the marker to the BAC(s) within the region. Touchdown PCR was as follows: 94 C for 5 min, then 10 cycles of 95 C for 1 min, 70 C for 30 sec decreasing by 1 C every cycle, and 72 C for 1.5 min. Then followed 25 cycles of 95 C for 30 sec, 55 C for 30 sec, and 72 C for 1.5 min and a final 72 C for 5 min. When more than two amplicons amplified with a single primer set from multiple BACs, the amplicon was sequenced and compared to the original contig to determine the correct BAC combination.

RESULTS

The 16 minimum tiling BAC contigs containing 36 clones identified for chromosome 2H bin 10 and a small part of the bin 9 region were divided into three groups of 12 each. Each group of 12 was sequenced in bulk at Washington State University using the 454 Life Sciences methodology. The sequence was delivered in computer assembled gene contigs, which were analyzed. Gene finder program Softberry (linux1.softberry.com) identified 129 putative genes of which 23 were previously known and mapped by us, not including the *Rrn5S1* gene. Based on recombinant analyses, we expect that the BF265762A to ctg15522 segment should contain the FHB resistance gene(s). We previously estimated this region to cover approximately 3,814 kb, not including the approximately 1,250 kb 5S RNA gene locus *Rrn5S1* present in this interval (Boyd et al., submitted).

Here we report more detailed analysis of Region 1 extending from BF265762A to BI948584 (Fig. 1). This approximately 1.2 cM region is covered by 6 BAC contigs represented by 12 minimum tiling BAC clones with a total minimum size of 1.39 Mb. Sequence analysis identified 50 putative genes, ten of which have been previously identified and mapped (Fig. 1). Gene numbers 30 and 32 come from the same sequence contig and identify the same EST, thus probably represent a recent duplication. Detailed characterization of the putative genes is shown in Table 1. Although some of the genes have putative functions assigned, for the most part they represent hypothetical proteins or have no significant homology (S value 80 or higher) in the NCBI database.

DISCUSSION

DNA sequencing using the newer sequencing technologies has become relatively easy and inexpensive. Taking advantage of the 454 sequencing technology available, we sequenced 36 BAC clones in 3 groups of 12 from the chromosome 2H bin 10 region presumed to contain the FHB resistance gene(s). Sequence analysis, however, is still a time consuming and hands-on process. Here we report the preliminary sequence analysis of Region 1 BAC clones, which includes the genomic region from marker BF265762A to BI948584. Although we had fairly saturated this region with markers, the number of putative genes identified exceeded our expectations, thus clearly illustrating the validity of the sequencing approach for gene discovery. Of the 53 putative genes identified by database searches only 22 have a putative function and several of these are listed as hypothetical proteins. Twenty-nine of the putative genes have "no significant similarity" in the NCBI database, defined as an S value below 80. It is quite probable that some of the putative genes are not real, but this is expected to be a relatively small portion of the total and might be offset by some genes that were missed by the gene finder program.

In summary, we have identified a large number of putative genes that reside in the chromosome 2H bin 10 region. Analysis of these putative genes will

facilitate completion of the BAC clone contigs to completely cover the chromosome 2H bin 10 region and lead to the identification of putative FHB resistance genes.

ACKNOWLEDGEMENT AND DISCLAIMER

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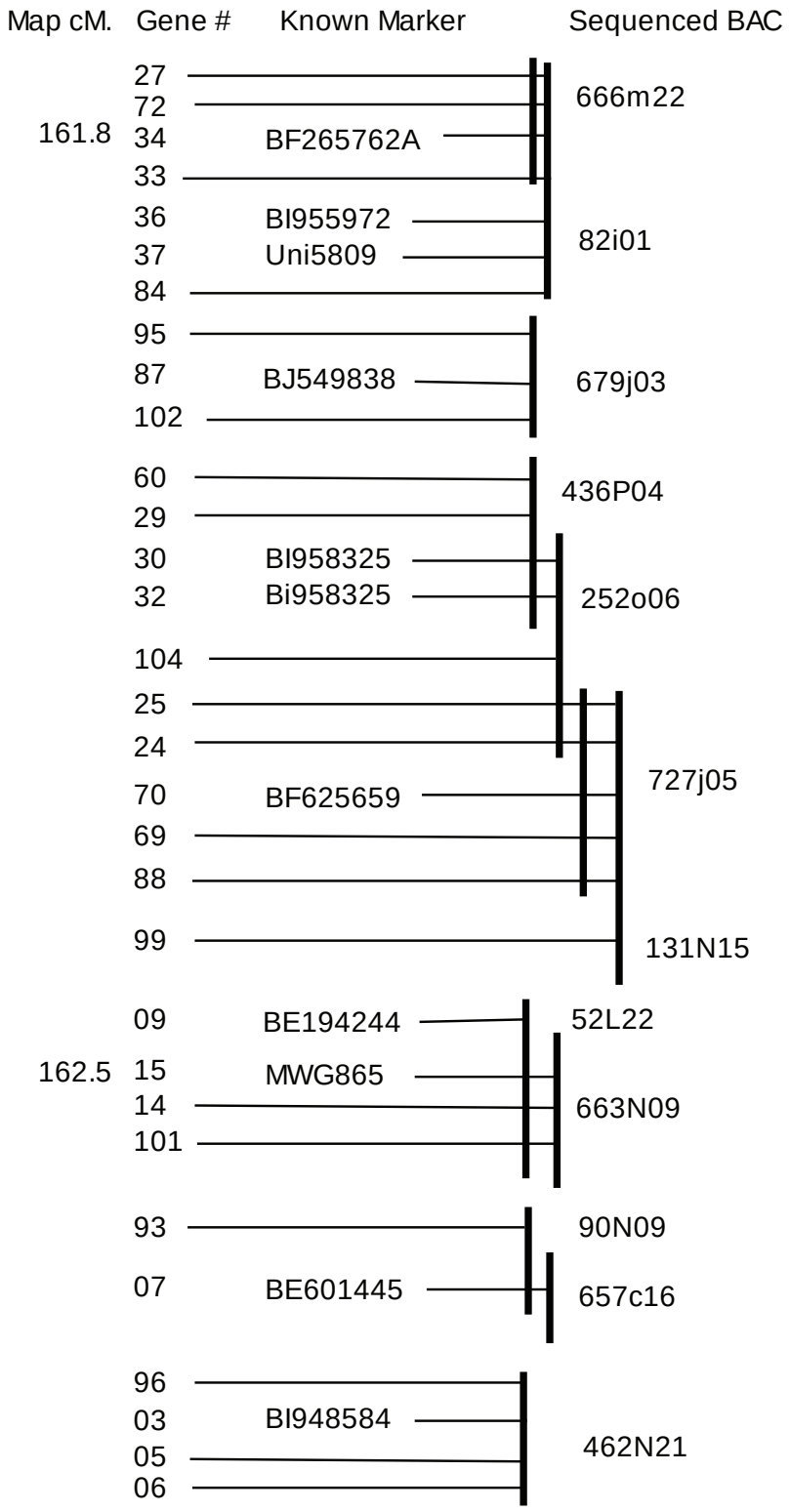

Fig. 1. Physical map location of predicted genes from chromosome 2H bin 10 Region 1. Known markers – previously mapped markers arranged in order from proximal to distal region. The unknown genes are arranged in a probable, but not confirmed, order based on their relationship with the known markers. Two cM values from mapped markers are indicated to facilitate orientation of the physical map. Gene # - is an arbitrary number assigned to predicted genes by the computer program used for analysis of the sequence. Sequenced BAC clones are identified. The predicted genes from Region 1 that are not yet assigned to specific BAC clones (see Table 1) are not included in this Figure.

Table 1. Chromosome 2H bin 10 Region 1 predicted gene BAC location, barley EST homology and putative function. Genes below #46 have not been localized to specific BAC clones at this time. Sequence contig number (seq. ctg#) refers to the contig assembled by the sequencing program software and an arbitrary number. Blastn hits on barley ESTs were scored as positive if the S value exceeded 80.

Region 1						
Gene#	seq ctg#	Known marker	Seq. BAC(s)	Blastn	Blastx	Score
72	12		82i01, 666M22	no hits	No significant similarity	
27	1732		82i01, 666M22	no hits	No significant similarity	
34	1366	BF265762A	82i01, 666M22	BF266945, BF265762	Os04g0542800 (Oryza sativa, Japonica)	540
33	1366			no hits	No significant similarity	
36	45	BI955972	82i01	BJ472255, BI955972	hypothetical protein OsJ_15640 (Oryza sativa, Japonica)	618
37	45	Uni5809		AJ462415	hypothetical protein SORBIDRAFT_06g024060 (Sorghum bicolor)	493
84	1415		82i01	no hits	No significant similarity	
95	1461		679j03	no hits	No significant similarity	
87	1470	BJ549838	679J03	CB869218, BJ549838	hypothetical protein OsJ_15627 (Oryza sativa, Japonica)	565
102	1811		679j03	BQ658680	Far1 [Triticum aestivum]	183
60	1550		436p04	GH220600	GATA zinc finger family protein (Zea mays)	253
29	1499			BY852017	No significant similarity	
30	1499	BI958325	436P04, 252o06	BI958325	hypothetical protein SORBIDRAFT_06g023950 (Sorghum bicolor)	302
32	1499	BI958325	436P04, 252o06	GH228028	OSJNBa0011L07.8 (Oryza sativa, Japonica)	283
104	1658		252o06	CB881126	ORF (Triticum aestivum)	123
25	1729		252o06, 131N15, 727j05	CX626672	No significant similarity	
24	1729		252o06, 131N15, 727j05	no hits	No significant similarity	
70	1437	BF625659	727j05, 131n15	BY847183, BF625659	H0115B09.3 (Oryza sativa, Indica)	264
69	1437			no hits	No significant similarity	
88	1858		727J05, 131N15	no hits	No significant similarity	
99	122		131n15	no hits	No significant similarity	
9	1485	BE194244	52L22	no hits	gt-2 (Oryza sativa, Indica)	262
15	1557	MWG865	52L22, 663N09	CB866125	UDP-glycosyltransferase UGT88C4 (Avena strigosa)	155
14	1557			BU992850	No significant similarity	
101	1766		52L22, 663N09	no hits	No significant similarity	
93	1537		90n08	no hits	No significant similarity	
7	111	BE601445	90n08, 657c16	CB883689, BE601445	Os04g0543200 (Oryza sativa, Japonica)	939
96	1819		462n21	BU986685	hypothetical protein OsI_17244 (Oryza sativa, Indica)	885
3	49	BI948584	462N21	GH224396, BI948584	Os04g0541900 (Oryza sativa, Japonica)	268
5	94		462N21	BF628929	putative far-red impaired response protein (Oryza sativa, Japonica	504
6	94		462N21-end	FD527594	hypothetical protein (Oryza sativa, Japonica)	103
46	41			no hits	No significant similarity	
47	41			no hits	No significant similarity	
48	41			no hits	No significant similarity	
75	60			no hits	No significant similarity	
55	126			BY840630	hypothetical protein OsJ_15628 (Oryza sativa, Japonica)	87
58	126			no hits	No significant similarity	
39	1450			BQ470966	hypothetical protein OsI_16839 (Oryza sativa, Indica)	885
40	1450			BG366371	No significant similarity	
11	1501			DN177342	RecName: Full=Formin-like protein 3; AltName: Full=OsFH3	587
13	1501			no hits	No significant similarity	
81	1543			no hits	No significant similarity	
64	1554			BU993407, BF618043	hypothetical protein (Beta vulgaris)	1001
43	1603			no hits	Os07g0285700 (Oryza sativa, Japonica)	147
66	1606			EX578159	forminy 2 domain-containing expressed protein (Oryza sativa, Jap	226
63	1608			CB865507	No significant similarity	
44	1630			CA014815	CK2 regulatory subunit B1 (Zea mays)	379
45	1630			no hits	hypothetical protein SORBIDRAFT_06g024070 (Sorghum bicolor)	874
52	1722			EX573578	No significant similarity	
50	1722			no hits	No significant similarity	

FIGHTING AGAINST FHB – AN EXCELLENT NOVEL RESISTANCE SOURCE FOR FUTURE WHEAT BREEDING

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ABSTRACT

Fusarium head blight (FHB) is a devastating wheat disease that causes tremendous economic losses by reducing grain yield and quality in wheat-growing areas worldwide. Sources of resistance are critical for genetically improving wheat for resistance to FHB. From a large-scale evaluation of tetraploid wheat (*Triticum turgidum*) germplasm for resistance reactions to FHB, we identified an accession (PI 277012) that consistently showed a high level of resistance across all environments in both greenhouse and field experiments. PI 277012 is currently classified as tetraploid emmer wheat (*T. turgidum* subsp. *dicoccum*) in the National Small Grains Collection, but somatic chromosome counts revealed that this accession was actually a hexaploid wheat. To characterize the FHB resistance in this accession, we developed a doubled haploid (DH) mapping population consisting of 130 lines from the cross between PI 277012 and the hard red spring wheat cultivar 'Grandin'. The DH population was then evaluated for reaction to FHB under three greenhouse seasons and five field environments. Based on whole genome linkage maps that consisted of 350 SSR markers spanning 2,703 cM of genetic distance, two major FHB resistance QTLs were identified on chromosome arms 5AS and 5AL. The 5AS QTL peaked at the marker interval between *Xbarc180* and *Xwmc795*, and explained up to 25% of the phenotypic variation. The 5AL QTL explained up to 35% of the trait variation and peaked at the interval between markers *Xwmc470* and *Xgwm595*. FHB resistance has not previously been reported to be associated with this particular genomic region of chromosome arm 5AL, thus indicating the novelty of FHB resistance in PI 277012. Furthermore, the FHB resistance effects of neither QTL were associated with plant height and maturity. Elite agronomic traits were observed among several FHB-resistant DH lines. Therefore, these results suggest that PI 277012 is an excellent source for improving FHB resistance in wheat. The markers identified in this research are being used for marker-assisted introgression of the QTLs into adapted durum and hard red spring wheat cultivars.

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USING DOUBLED HAPLOIDS TO SPEED UP GENETIC ANALYSIS FOR RESISTANCE TO FHB AND OTHER COMPLEX TRAITS IN WHEAT

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ABSTRACT

Doubled haploids (DHs) or recombinant inbred lines (RILs) are useful in wheat for analyzing resistance to Fusarium head blight (FHB) or other complex traits that are governed by multiple genes and/or subject to environmental influences. This is because DHs or RILs allow highly-confident assessment of phenotypic differences in replicated trials under different conditions. DHs, obtained by doubling the chromosome number of haploids, are genetically pure and obtained in a single generation. This makes DHs much better for genetic analysis than RILs, which take far longer to produce and contain some residual heterozygosity. The complete homozygosity of DHs enables accurate evaluation of genetic effects, thus facilitating identification of genes controlling a complex trait such as resistance to FHB. Highly-efficient use of DHs in identifying novel gene sources for FHB resistance or other complex traits in wheat has recently been demonstrated. However, DHs have not been widely used in public wheat breeding programs in the United States mainly due to the complexity of producing DHs. In order to produce DHs on a scale that meets the requirements of wheat breeders, Heartland Plant Innovations (HPI) Inc. has launched a doubled haploid laboratory devoted to providing DHs on a fee-for-service basis for both public and private customers. Located on the campus of Kansas State University in Manhattan, Kansas, HPI is a unique collaboration of public and private partners consisting of a team of agricultural producers, public research institutions and plant science technology companies. Besides providing DH service for wheat breeders and geneticists, the DH lab in HPI will also focus on improving methods for highly-efficient production of wheat DHs, with the goal of greatly reducing the cost of wheat DH lines.

TESTING TRANSGENIC SPRING BARLEY LINES FOR REACTION TO FUSARIUM HEAD BLIGHT: 2010 FIELD NURSERY REPORT

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ABSTRACT

The 2010 field screening nursery, with 88 barley plots was located at UMore Park, Rosemount MN. Trial entries (n=18 transgenic) and an untransformed 2-row control Conlon (susceptible) were submitted by USDA-ARS, RRVARC Fargo. Barley lines with known reactions to Fusarium head blight (FHB) were also included as checks. The checks used were the moderately resistant cultivar Quest (included in previous nurseries as breeding line M122) and the susceptible cultivars Robust and Stander. The experimental design was a randomized block with four replicates. Plots were 2.4 m long single rows. The trial was planted on May 4, 2010. All plots were inoculated twice, with the first inoculation applied at head emergence. The second inoculation was applied three days after the initial inoculation (dai) for each plot. The inoculum was a composite of 51 *F. graminearum* isolates at a concentration of 200,000 macroconidia ml^{-1} with Tween 20 (polysorbate) added at 2.5 ml L^{-1} as a wetting agent. The inoculum was applied at a rate of ca. 30 ml per meter of plot row. The inoculum was applied using a CO_2-powered backpack sprayer fitted with a SS8003 TeeJet spray nozzle with an output of 10ml sec^{-1} at a working pressure of 275 kPa. Mist-irrigation was applied from the first inoculation on June 28 till July 15 to facilitate FHB development. FHB incidence (FHBI) and severity (FHBS) were assessed visually 14 dai on 20 arbitrarily selected spikes per plot. FHBI was determined by the percentage of spikes with visually symptomatic spikelets of the 20 spikes observed. FHBS was determined as the percentage symptomatic spikelets of the total of all spikelets observed on the 20 spikes. Plots were harvested at maturity on August 5. The harvested seed from each plot was split to obtain a 25 g sub-sample, which was then cleaned by hand. The samples were ground and submitted for deoxynivalenol (DON) analysis. FHBI for all treatments ranged from 86 to 99%. FHBS ranged from 13 to 36% for the 18 entries examined. The FHBS for the untransformed control Conlon was 23%. The FHBS for the moderately resistant check Quest was 15% while FHBS for the susceptible checks Robust and Stander were 15% and 22%, respectively. The level of disease was similar to the 2009 nursery. We anticipate the DON data (not yet available) will provide additional information on the response of these entries to FHB.

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CHROMOSOME ENGINEERING AND TRANSFER OF ALIEN SOURCES FOR FUSARIUM HEAD BLIGHT RESISTANCE IN HARD RED WINTER WHEAT

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ABSTRACT

We report on progress made in incorporating two new sources of resistance to Fusarium head blight (FHB) from *Leymus racemosus* and *Elymus tsukushiense* to hard red winter wheat. FHB resistance gene *Fhb3* from *L. racemosus* was transferred to wheat in the form of a compensating Robertsonian translocation T7AL·7Lr#1S. The *Fhb3* gene is located on the short arm of *L. racemosus* chromosome 7Lr#1S translocated to the long arm of wheat chromosome 7AL. The 7AL and 7Lr#1S arms are joined at the centromere. *Fhb3* confers resistance to single-point inoculation in the greenhouse. Ten lines homozygous for *Fhb3* in Jagger and Overley background were evaluated for their resistance to FHB and DON accumulation under field conditions in the 2008-9 growing season. Although the lines were homozygous for *Fhb3*, variable reaction to FHB and DON accumulation was observed, which could be caused by genetic background effects. Lines 08-193 (in Jagger) and 08-184 (in Overley) with higher levels of resistance were evaluated in the field FHB nursery in Manhattan in the 2009-10 growing season. The line 08-193 gave a disease index rating of 27.6% as compared to 36.8% for Jagger. The line 08-184 gave an index rating of 33.1% as compared to 50.2% for Overley. Both of these differences were significantly different ($P<0.05$, LSD=5.66). For further evaluating the genetic background effects we are backcrossing 08-193 to the cultivar Fuller. After a second backcross to Fuller, the selfed progenies will be screened for isolating homozygous *Fhb3* lines and will be evaluated for FHB resistance and DON accumulation under greenhouse and field conditions.

Chromosome engineering was used to isolate three recombinant chromosomes, one proximal rec124 (T7AL·7Lr#1S-7AS), and two distal rec989 and rec679 (T7AL·7AS-7Lr#1S) and homozygous lines were developed in Overley background. In the 2009-10 growing season, all the recombinant lines along with Overley were evaluated for FHB and DON. Rec124 had an FHB index rating of 27.6% as compared to rec679 (38.1%), rec989 (48.8%) and Overley (50.2%; LSD=5.66). DON ranged from 7.4 to 14.6 ppm as compared to 19.7 ppm for Overley (LSD=4.22). It appears that based on this and previous data, *Fhb3* is located in the interstitial region (FL 0.45-0.80) of the 7Lr#1S arm.

Another source of FHB resistance is derived from *Elymus tsukushiense* and was transferred to wheat in the form of a disomic chromosome addition stock (DA1Est#1), a ditelosomic addition stock (DtA1Est#1S), and a disomic addition/translocation stock DATW1Est#1S. Testing of the DATW1Est#1S stock from 2005 to 2007 indicated that this line conferred resistance to FHB under greenhouse conditions. In a spring 2010 greenhouse test, the lines T7AL·7Lr#1S (*Fhb3*), DA1Est#1, DtA1Est#1S, and DATW1Est#1S gave average spike index ratings of 19.2%, 26.2%, 23.5%, and 10.0%, respectively, whereas the resistant check Sumai#3 and the moderate susceptible check Chinese Spring had ratings of 5.3% and 32.0%, respectively (LSD=9.05). We are presently using directed chromosome engineering to produce recombinants with shortened *E. tsukushiense* chromatin, which will then be evaluated for their resistance to FHB and DON accumulation.

PUTATIVE ROLE FOR ETHYLENE SIGNALING IN TYPE II RESISTANCE TO *FUSARIUM GRAMINEARUM* IN WHEAT USING VIRUS INDUCED GENE SILENCING (VIGS) AND A GASEOUS INHIBITOR OF ETHYLENE PERCEPTION

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ABSTRACT

Ethylene (ET) has been shown to be important for resistance to necrotrophic pathogens in Arabidopsis. While it remains unclear as to whether *Fusarium graminearum* is a hemibiotroph or a necrotroph, its necrotrophic mode of growth is most damaging. Thus, ET is a potential candidate for disease resistance signaling. We have used a Virus-Induced Gene Silencing (VIGS) system to silence genes in both the ethylene biosynthesis pathway and the ethylene signaling pathway. Preliminary results indicate that a number of these genes may indeed be important for defense signaling against *Fusarium graminearum*. The genes were silenced in the resistant variety 'Ning' 7840. Upon application of the virus, containing a portion of a wheat gene, the plants were screened for conversion from resistance to susceptibility. The genes involved in ET signaling screened thus far include SAMs, ACS, ETO, CTR, EIN2, and an ERF. SAMs and the ERF in particular demonstrated remarkable conversion to susceptibility upon silencing. The importance of ethylene signaling in the resistant genotype was also observed using the gaseous inhibitor of ethylene signaling 1-Methylcyclopropene (1-MCP). Ning plants exposed to this inhibitor became significantly more susceptible to Fusarium than control plants.

CHARACTERIZATION OF FUSARIUM HEAD BLIGHT-RESPONSIVE GENES IN DIVERSE WILD AND CULTIVATED BARLEY

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ABSTRACT

Wild (*Hordeum vulgare* subsp. *spontaneum*) and cultivated barley (*Hordeum vulgare*) accessions possess various degrees of resistance to Fusarium head blight (FHB). Integration of resistance from diverse sources has the potential to enhance resistance, ultimately helping barley producers manage FHB. To identify genetically diverse barley lines carrying FHB resistance, DArT markers were used to genotype 102 wild or cultivated barley lines (80 FHB-resistant and 22 FHB-susceptible). Population structure was analyzed using two methods. First, phylogenetic analysis was conducted with MrBayes. Second, software STRUCTURE was used to infer distinct genetic populations in the germplasm collections. Both methods suggest the presence of four populations which comprised : (1) spring six-row, (2) spring two-row, (3) winter six-row and (4) wild two-row. Multiple wild and cultivated lines, including parents of mapping populations, were selected from across these major groups for haplotype and association mapping analysis. Previous GeneChip experiments have identified over 100 barley genes with significantly up-regulated transcript levels in response to treatment with *Fusarium graminearum* or DON. Thirty-nine of these genes, including those implicated in defense responses such as P450s, glutathione-S-transferases, and UDP-glucosyltransferases, have been sequenced from 24 diverse barley lines (13 resistant and 11 susceptible) and analyzed using an association-based approach. Seventeen of these genes were genetically mapped using the Oregon Wolfe Barley population. Map locations of twelve genes are within previously identified FHB QTLs. Whole genome and gene-based association mapping identified associations that are coincident with previous reported FHB QTL regions as well as potentially novel locations. The DArT marker haplotype diversity at FHB resistance QTL regions was analyzed and will be reported.

COMPREHENSIVE METABOLOMICS AND PROTEOMICS FOR FUSARIUM HEAD BLIGHT RESISTANCE GENE DISCOVERY AND FUNCTION IN TRITICEAE

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OBJECTIVES

To identify mechanisms of resistance in Triticeae against *Fusarium graminearum*, through mass spectrometry based comprehensive metabolomics and proteomics.

INTRODUCTION

The resistance in Triticeae (wheat, barley and triticale), to fusarium head blight (FHB, scab) caused by *Gibberella zeae* (anamorph: *Fusarium graminearum*), is quantitatively inherited and is controlled by several genes (3). The resistance mechanisms in Triticeae to FHB can be apparent or true. The cleistogamous florets in two row-barley lead to apparent resistance while the chasmogamous florets in six-row barley and wheat lead to susceptibility. In the chasmogamous floret genotypes, unlike in cleistogamous floret genotypes, the biochemical resistance in lemma and palea do not serve as a barrier that prevents the pathogen from attacking the ovary during anthesis. Excellent work has been done on apparent resistance including discovery of genes for cleistogamy. Other apparent resistance mechanisms include occurrence of less favourable environment for infection by pathogen due to plant height, spikelet density, etc. which mainly reduce duration of wetness. The true resistance is mainly due to structural or biochemical, which can be either constitutive or induced following pathogen inoculation. The biochemicals may be either proteins/enzymes/peptides or metabolites. Breeding for resistance requires high throughput selection parameters. Accordingly, the resistance is classified into type I = resistance to initial infection and type II = resistance to spread of infection within spike. Barley has very high type II resistance, while wheat varies among genotypes. More than 100 FHB resistance quantitative trait loci (QTLs) have been identified, in all the seven chromosomes, but only one third of these are stable and/or not associated with apparent resistance (3). The mechanism of resistance has been partially elucidated for one QTL on chromosome 3BS, which is due to an enzyme, DON-3-*O*-glucosyltransferase that converts the virulence factor DON to DON-3-O-glucoside (D3G) (7); the trichothecene lacking mutants fail to progress to the rachis in wheat. Though the cultivar ranking for type II resistance based on individual floret inoculation is quite consistent, the ranking of type I resistance is very inconsistent over years and seasons. Though marker assisted selection facilitates plant breeding it is often associated with apparent resistance mechanisms and the true mechanisms are not revealed. It is also possible that a given resistance mechanism can be controlled by more than one QTL. Thus, molecular breeders are looking for novel tools which also would lead to the identification of genes. In this study, our focus was to apply mass spectrometry based comprehensive metabolomics and proteomics to better understand the mechanisms of resistance in Triticeae against FHB.

MATERIALS AND METHODS

Comprehensive metabolomics and proteomics of biotic stress involved ten steps:

Step 1. Plant-pathogen systems for comparison: Metabolic and proteomic profiles of resistant and susceptible plant genotypes, inoculated with mock and pathogen, were compared. However, the effect of genetic background was not controlled. To address this problem, several genotypes and pairs of near isogenic lines (NILs) with FHB resistance QTLs were compared, as a precursor to evaluation of a population of recombinant inbred lines (RILs). Additionally, further protocol standardization is required. Use of cultivars, with several mechanisms of resistance, can help detecting several known mechanisms of resistance. Transgenic mutants lacking one target gene would also be ideal. However, not all the mechanisms are controlled by biochemicals. Other mechanisms also must be studied.

Step 2. Plant and pathogen production and inoculation conditions: Plant-pathogen interaction to produce disease depends on three components of disease triangle: host, pathogen and environment. Plants varying in resistance to FHB were produced under greenhouse conditions to reduce temperature variation. A single spore isolate of *F. graminearum* was used to avoid mixture of chemotypes. Trichothecene non-producing mutants were used to investigate mechanisms not involving trichothecenes. Spikelets were spray inoculated to assess spikelet resistance, and single spikelets were inoculated to assess disease spread within a spike (4).

Step 3. Assessment of resistance in plants against biotic stress: The number of spikelets diseased at 3d intervals until 21 dpi was assessed. The proportion of spikelets diseased (PSD) on 21 dpi or area under the disease progress curve (AUDPC) was calculated (4). The experimental design was a randomized complete block design with blocks over time of 3-5 d. Up to 30 spikelets were used to assess disease severity. Pair (Res. vs Sus.) wise comparison based on students *t*-tests, or grouping based on canonical discriminant analysis (CDA), using SAS (4) were performed.

Step 4. Sample collection for biochemical analysis: Spray or single spikelet inoculated, spikelets or rachis, at GS=65 or 75, from mid third of spike, were harvested at 48-72 hpi. Lemma+palea, lemma+palea+caryopsis or rachis were separately placed in vials, liquid nitrogen was added and stored at -80C. The experiment was conducted as a RCBD with five blocks over time. Each experimental unit consisted of 50-60 spikelets or 10-15 rachises.

Step 5. Metabolite and protein extraction: Samples were crushed in liquid nitrogen, 100 mg samples were taken, internal standards were added, extracted with methanol+water, and centrifuged (1). For GC/MS analysis the polar and apolar metabolites were separated using chloroform. The pellet was used for protein extraction and digested with tripsin to breakdown the proteins into peptides (5).

Step 6. Mass spectrometer analysis: Liquid chromatography and hybrid mass spectrometry (LC-nESI-LTQ-Orbitrap) was used for metabolite and protein/peptide analysis. Polar and semipolar columns were used. Metabolites were analyzed in negative ion mode at 60 000 resolution (at m/z = 400). Peaks were fragmented using 35 eV CID energy (1).

Step 7. Mass spectral output processing: The output from LC/MS was converted to netCDF using Bioworks (Thermos), imported to XCMS for deconvolution and alignment of peaks across samples. Peaks with signal to noise ratio s/n < 5 were used to avoid higher probability of extraction of peaks with high background spectra. Peaks with adducts and isotopes were identified using CAMERA, sieved using MS-EXCEL (1). The output from GC/MS was converted to netCDF using MassLynx, imported to AMDIS and peaks deconvoluted. Peaks were aligned and the abundance was calculated using MetIdea (4).

Step 8. Information extraction: Abundances of peaks (metabolites and peptides) were subjected to *t*-tests to sieve treatment significant peaks: RP

vs RM, SP vs SM, RP vs SP and RM vs SM, where R=resistant, S=susceptible, M=mock inoculated and P=pathogen inoculated. These were further subjected to canonical discriminant analysis (CDA) to cluster observations. CDA uses first a non-supervised principal component analysis to cluster observations and then a supervised analysis to reduce the distance within clusters. The CAN-vector scores and their associated loading to metabolites/proteins/peptides were used to identify the biomarker metabolites (1, 4).

Identification of pathogenesis (PR) and resistance related (RR) metabolites/proteins: *t*-test was used to identify PR and RR metabolites/proteins. Biochemicals with higher abundance in pathogen than in mock inoculated were designated as PR, and those in resistant genotype as PRr (RP>RM) and in susceptible as PRs (SP > SM). Biochemicals with higher abundance in resistant than in susceptible genotypes were designated as RR, and those based on mock inoculation were designated as constitutive (RRC = RM > SM) and on pathogen inoculated as induced (RRI = RP > RM and RP > SP) (1, 4). The median accurate masses obtained from XCMS linked to METLIN which was further linked to various databases (8) were used to putatively identify metabolites. Metabolites were identified based on three criteria: i) accurate mass (AME<5ppm), ii) isotope ratio based number of carbons in the formula; iii) fragmentation pattern using InteliXtract (ACDlab, Torornto). Some of these metabolites were matched to purified metabolites. The GC/MS peaks were identified based on fragmentation patterns (4). The peptides were imported to Mascot and the proteins were identified (Raghavendra – unpublished).

Identification of resistance indicator induced (RII) metabolites and proteins: The trichothecenes produced by the pathogen in the plant, especially the virulence factors, and their degradation products as a result of enzymes produced by resistant hosts were designated here as RII metabolites. Similarly, the enzymes that catalyze reactions of RR metabolites were designated as RII proteins/enzymes as they are not directly involved in resistance.

Step 9. New knowledge generation and its validation: The PR and RR biochemicals identified here were linked in their metabolic pathway to identify the precursors and the end products. These were searched in the literature for their role in plant defence, as plant metabolomics may be new but not the metabolites. For some RR metabolites the ability to reduce biomass was determined. This was combined with the abundance of the compound in host to derive resistance equivalence (RE).

Step 10. Application of knowledge and technology transfer: Use of RR metabolites and proteins in crop improvement require cost effective tools. The potential use of HPLC, qRT-PCR, molecular markers, etc., will be discussed.

RESULTS AND DISCUSSION

Disease severity: In spray inoculated barley the PSD and AUDPC varied significantly among genotypes tested. Barley has high type II resistance, however, in wheat the PSD varied among genotypes. For the evaluation of type I resistance, inoculation of spikelets during anthesis is not a suitable growth stage for chasmogamous florets as the pathogen avoids biochemical resistance in glume, lemma and palea. Inoculation at later stages can reveal resistance in caryopsis.

Metabolic and proteomic profiles: Metabolomic and proteomic/peptide profiles yielded more than 3000 peaks. About 1000 metabolites had significant treatment effects. These were further subjected to univariate and multivariate analyses. Peptides were used to identify proteins (analysis in progress).

PR/RR metabolites and their functions: The CDA of the abundances of treatment significant metabolites discriminated resistant and susceptible cultivars. In general the CAN vectors discriminated constitutive resistance and pathogenicity functions. Induced resistance was not well separated (1)

More than 300 metabolites were identified as PR or RR metabolites. Out of these about 200 metabol-

ites were putatively identified (Table 1). In general, more metabolites were RRC and very few were RRI, meaning barley genotypes have constitutive resistance to defend against this necrotrophic pathogen. However, the use of Tri5- mutant increased RRI metabolites, meaning otherwise the enzymatic reactions were prevented by DON. Biomass inhibition and the resistance equivalence of RR metabolites varied. The RR metabolites identified here belonged to mainly three metabolic pathways: phenylpropanoid-flavonoid, terpenoid and fatty acid pathways. These metabolites are known to have several plant defence functions, including pathogen biomass reduction, trichothecene toxin synthesis inhibition, signalling, etc. In addition, trichothecenes and their degradation products were detected and these are discussed below, under RII metabolites.

Phenylpropanoid and flavonoid pathway - Phenylpropanoid and flavonoid or phenyl ammonia lyase (PAL) pathway is activated through over expression of PAL enzymes. Several phenylpropanoids were RR metabolites, including phenylalanine, *p*-coumaric acid, sinapate, ferulic acid, etc. Among the monolignols (*p*-coumaryl, coniferyl, and synapyl alcohols) only coniferyl alcohol was detected, though several lignans, such as diphyllin, phyllanthin, eucommin A, and tuberculatin, were detected (6). The flavonoids that were RR metabolites were: kaempferol along with several of its conjugates, quercetin, naringenin, catechin, etc. The phenolics are known for their antioxidant properties and also lead to the formation of lignin which acts as a cell wall barrier to pathogen penetration. Though only ferulic acid has been shown to inhibit synthesis of trichothecene toxins (2), it is possible that several other phenolic and other compounds with antioxidant properties would also be able to inhibit toxin synthesis by the pathogen. These phenolic and flavonoid compounds are also known for their antimicrobial properties (1, 2). Accordingly, the metabolite abundance and its antimicrobial property were combined to derive resistance equivalence (1).

Terpenoid pathway - These metabolites contain isoprene units and are produced through farnesyl diphosphate downstream to the mevalonate pathway. Several mono, di, and tri-terpenes are known for their antimicrobial activities. Those identified as RR metabolites in barley against FHB include rishitin, polygodial, polygonic acid, boschnialactone, thymoquinol dimethyl ether, and cuauhtemone (1, 6).

Fatty acid pathway - Also known as octadecanoic acid pathway, this pathway leads to the production of jasmonic acid (JA), an important signal molecule. In barley, several precursors of JA, including linoleic and linolenic acid were produced, and all had high antimicrobial activity. In addition, capric acid and lauric acid were detected, and the capric acid was the most effective among the 20 RR metabolites evaluated.

PR and RR proteins and their functions: A preliminary proteomics analysis of wheat rachis detected more than 3000 peaks of peptides and identified about 100 proteins, including several PR proteins (Raghavendra - comparative analysis is in progress).*RII metabolites and their functions:* DON and 3ADON, along with their acetate adducts, were the only trichothecenes detected in this barley study. The plant enzymatic DON conversion product, D3G was also detected along with its acetate adduct. In addition, a novel plant enzymatic conversion product, S-methyl-DON (SMD) was detected, both in barley and in wheat. This can be traced back to the responsible enzyme and gene, and lead to the discovery of a novel gene. Total DON produced (TDP) and the proportion of total DON converted (PTDC) to D3G and also to SMD can be used as biomarkers in the evaluation of breeding lines. The TDP was the lowest in barley CIho 4196, and this could be due to the inhibition of trichothecenes through antioxidant properties of phenylpropanoids (2). The PTDC was the highest in Zhedar-2 and moderate in CIho 4196, though even a susceptible genotype had PTDC=0.4 (6). However, our proteomic analysis has yet to detect these enzymes. This indicates that PTDC is not the sole mechanism for type II resistance. *Future applications:* The RR metabolites identified here, and also the RR proteins to be identified, can be used as biomarkers to screen breeding lines, following validation of the occurrence of these biochemicals in other resistant genotypes. Most of the RR metabolites identified here have small and additive effects; thus, demonstrating their role indi-

vidually is difficult. Mass spectrometry technology is not suitable for plant breeding as it is expensive However, it could be used to establish the functions of the many QTLs already identified and lead to simpler methods suitable for the selection for disease resistance characteristics used for crop improvement.

Mass spectrometry for biochemical analysis is a powerful tool. With the advent of genome sequencing, including wheat and barley, MS can be used for functional genomic studies. Following gene identification, more specific molecular markers can be identified and used in trait transfer to elite cultivars. Additionally, the knowledgebase generated here can be used in metabolic pathway engineering.

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Table 1. Resistance related (RR) and resistance indicator induced (RII) metabolites in barley-Fusarium interaction detected based on LC hybrid MS.

Chemical group	RR and RII metabolites
Phenolics	RR: phenylalanine, *p*-coumaric acid, sinapate, ferulic acid, diphyllin, phyllanthin, eucommin A, tuberculatin, coniferyl alcohol, syringetin 3-rutinoside, quinic acid, scoparin
Flavonoids	RR: quercetin, naringenin, catechin, kaempferol 3-O-rhamnoside, naringenin 7-glucoside
Terpenes	RR: rishitin, polygodial, polygonic acid RII: deoxynivalenol, DON-3-*O*-glucoside, S-methyl-DON
Fatty Acids	RR: jasmonic acid, capric acid, lauric acid, linolenic acid, undecanoic acid

ACTIVATION TAG SCREENING TO IDENTIFY NOVEL GENES FOR TRICHOTHECENE RESISTANCE

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ABSTRACT

The goal of our research is to identify plant genes which enhance trichothecene resistance and, ultimately, Fusarium Head Blight resistance in wheat and barley. We are taking a two pronged approach using *Arabidopsis* to identify plant genes which confer resistance to trichothecenes. The first approach identified *Arabidopsis* orthologs of previously identified yeast knockouts from a viability screen using a trichothecene mycotoxin, trichothecin. An alternative method to identify trichothecene targets in plants is to perform mutagenesis followed by selection. T-DNA mutagenesis coupled with phenotypic selection has proven to be an extremely successful strategy to identify and isolate genes. One drawback from screening traditional T-DNA mutants is the problem of gene redundancy whereby knockouts do not present identifiable phenotypes. An alternative version of T-DNA mutatgenesis, termed activation tagging, provides an effective approach to overcome this limitation. Activation tagging uses a modified T-DNA vector which contains multiple copies of the cauliflower mosaic virus (CaMV) 35S gene enhancer arranged in tandem. In addition to knocking out genes, the modified T-DNA vector can also function as an enhancer when inserted either upstream or downstream of a gene to produce gain-of-function phenotypes. The genomic location of the tag is readily identifiable by thermal asymmetric interlaced (TAIL) PCR. Using this approach, we have screened >45,000 activation tagged *Arabidopsis* seeds for resistance to trichothecin and identified 15 lines that showed a very high level of resistance. These plants were able to form roots on 4 μM Tcin, a concentration which severely inhibits germination and prevents root formation of the Col-0 wild type. We will present the preliminary characterization of two of these mutants. Sequence analysis of the resistant lines by TAIL-PCR demonstrated T-DNA insertions in two novel genes, termed *Arabidopsis thaliana resistant root formation1* and *5* (*AtTRRF1* and *AtTRRF5*). *Arabidopsis* plants with independently generated knockouts (T-DNA) in these two genes are currently being tested for resistance. In addition, we are testing expression of neighboring genes by qPCR for upregulation due to the enhancer sequences. We propose that screening a large activation tagged *Arabidopsis* collection on media containing trichothecene mycotoxins provides an extremely flexible and efficient method to identify novel genes for trichothecene resistance in plants.

ENGINEERING DEFENSE REGULATORY GENES AND HOST SUSCEPTIBILITY FACTORS FOR ENHANCING FHB RESISTANCE

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ABSTRACT

Fusarium head blight (FHB)/scab caused by the fungus *Fusarium graminearum* is a destructive disease of wheat and barley. Due to the lack of monogenic gene-for-gene resistance to FHB, the mechanism(s) involved in signaling and activation of plant defense against *F. graminearum* are poorly understood. The utilization of a host-fungus system consisting of *Arabidopsis* and *F. graminearum* has provided an excellent model system to identify and characterize defense regulatory genes and genes that contribute to host susceptibility to *F. graminearum* that could be targeted for enhancing FHB resistance in wheat. Salicylic acid (SA) signaling was previously shown to promote resistance against *F. graminearum* in Arabidopsis (Makandar et al. 2010) and overexpression of the *NPR1* gene, which is a key regulator of SA signaling, was shown to enhance resistance against *F. graminearum* in Arabidopsis and wheat (Makandar et al., 2006, 2010). This interaction between Arabidopsis and *F. graminearum* has been utilized to identify additional genes (*PAD4*, *WRKY18*, and *LOX*s), that offer promising targets for enhancing FHB resistance in wheat. *PAD4* regulates multiple defense mechanisms, including SA synthesis and signaling in Arabidopsis, and *WRKY18* encodes transcription factor that regulates defense gene expression. In contrast to *NPR1*, *PAD4*, and *WRKY18*, which promote defense against *F. graminearum*, a lipoxygenases activity contributes to host susceptibility to this fungus. To determine if altered expression of these genes can promote FHB resistance in wheat, we have generated transgenic wheat plants that constitutively express *PAD4* and *WRKY18* from the ubiquitously expressed *Ubi* promoter. In addition, transgenic wheat plants that are silenced for expression of various lipoxygenases encoding genes have also been generated. Results on the analysis of these plants will be presented. In addition, progress on targeting non-host resistance mechanism for engineering FHB resistance in wheat will also be presented.

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DEVELOPMENT AND TESTING OF IMPROVED ENZYMES FOR TRANSGENIC CONTROL OF FHB

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ABSTRACT

The primary goal of the present study is to develop improved enzymes for the inactivation of trichothecene mycotoxins associated with Fusarium head blight and test their efficacy in barley. Trichothecene mycotoxins such as DON play a prominent role in the establishment of FHB and have been implicated in pathogen virulence. A primary agent for the inactivation of trichothecene mycotoxins is the trichothecene 3-*O*-acetylase (TRI101) enzyme. TRI101 catalyzes the acetylation of the 3-OH on the trichothecene toxin resulting in a 100-fold decrease in toxicity. Therefore efforts to use TRI101 from *Fusarium sporotrichioides* as a transgenic resistance factor have been implemented in wheat (*Triticum aestivum)*, barley (*Hordeum vulgare*), and rice (*Oryza sativa*). These transgenic cereals have shown moderate resistance to FHB in greenhouse tests, but have shown little success in field trials. *In vitro* kinetic analysis of the TRI101 enzymes from *Fusarium sporotrichioides* (FsTRI101) and *Fusarium graminearum* (FgTRI101) reveal that FgTRI101 has 100-fold greater efficiency (k_{cat}/K_M) for the acetylation of DON. It is proposed that this significant kinetic difference accounts for the poor performance of transgenic cereals in field trials. Consequently the present work is focused on optimization of the kinetically superior FgTRI101 for expression in barley. The 3-dimensional structure of FgTRI101 was used to engineer several point mutations to improve the stability and solubility of the enzyme in its transgenic host. Strategies such as entropic stabilization, consensus mutagenesis, and surface charge introduction were employed to create an optimized FgTRI101. An increase of 4.7 °C in enzyme melting temperature and a catalytic efficiency comparable to the wild type FgTRI101 were observed for the optimized enzyme. Both the wild type and optimized FgTRI101 have been inserted into plasmid pBract214 and have been utilized in *Agrobacterium*-mediated transformation of barley to create transgenic strains. Transformed plants have been obtained and will be analyzed for resistance to FHB once homozygous lines are identified. Additionally, an antibody-based purification protocol has been established for TRI101 expressed in transgenic barley. This protocol has been used to isolate FsTRI101 from barley and has shown that the transgenic enzyme has retained enzymatic activity although western blots indicate that the enzyme has been post-translationally modified. Future studies will examine the nature of this modification and also characterize the optimized and wild type FgTRI101 enzymes from transgenic barley. These studies will establish a connection between the *in vitro* and *in vivo* studies of TRI101.

GREENHOUSE EVALUATION OF TRANSGENIC BARLEY EXPRESSING *GASTRODIANIN* FOR RESISTANCE TO FUSARIUM HEAD BLIGHT

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OBJECTIVE

To develop transgenic barley expressing *gastrodianin* for resistance against *Fusarium* head blight (FHB).

INTRODUCTION

The filamentous ascomycete *Fusarium graminearum* Schwabe [teleomorph *Gibberella zeae* (Schwein) Petch] is the major pathogen causing Fusarium head blight (FHB) in barley, wheat, oats and other cereals (McMullen *et al*., 1997). FHB-infected plants have reduced yield due to sterile florets and shriveled kernels. Furthermore, infected kernels are contaminated with trichothecene mycotoxins such as deoxynivalenol (DON) and 15- acetyldeoxynivalenol (15-ADON; Desjardins, *2006*) that are harmful to humans and animals (Rocha *et al*., 2005).

Barley has very limited resistance to FHB (Bai and Shaner, 2004). FHB resistance in barley primarily involves restriction of initial infection known as type I resistance (Steffenson, 2003). Quantitative trait loci (QTLs) for FHB resistance often map to the location of other QTLs including those for heading date, plant height, spike angle (Bai and Shaner, 2004), and two-row spike type (Mesfin *et al*., *2003)*. This relationship between QTLs for FHB resistance and other traits is due to tight linkage rather than a single QTL controlling several phenotypes (Nduulu *et al*., *2007*). Identification of resistance QTLs is pivotal for the development of germplasm with improved resistance to FHB through breeding. Advances in genome-wide association mapping may facilitate development of effective markers for breeding resistant barley (Massman *et al*., 2010). However, progress in breeding resistant lines has been slow. Introduction of anti-*Fusarium* genes through genetic engineering may complement breeding efforts to enhance resistance to FHB.

We have transformed barley with *gastrodianin*, an anti-fungal gene isolated from *Gastrodia elata,* for resistance against FHB. Gastrodianin is a 12 kDa, non-agglutinating, monomeric, mannose and chitin-binding lectin that belongs to the superfamily of monocot mannose-specific lectins (Wang *et al*., 2001). *In vivo* studies have established that gastrodianin inhibits the growth of saprophytic fungi including *F. graminearum* (Wang *et al*., 2001). *In vitro* studies using transgenic tobacco and plum showed that gastrodianin inhibits root rot caused by the fungal pathogens *Rhizoctonia solani, Phytophthora nicotianae* and *P. cinnamomi* (Cox *et al*., 2006; Nagel *et al*., 2008). Field tests in cotton have also demonstrated that transgenic plants expressing *gastrodianin* are more resistant to *Verticillium* wilt (Wang *et al*., 2004). The role of gastrodianin in fighting fungal pathogens is probably attributable to its ability to bind to fungal cell walls and slow hyphal growth. Gastrodianin is stable at fluctuating temperatures (Wang *et al*., 2001). Its stability and inhibitory effects on fungal pathogens makes *gastrodianin* an attractive gene for engineering resistance to FHB. In this study we report preliminary results in the response of transgenic barley expressing the *gastrodianin* gene against *F. graminearum* infection under greenhouse conditions.

MATERIALS AND METHODS

Transformation and Cytological Analysis of Transgenic Plants - Immature barley (*Hordeum vulgare* cv. Golden Promise) embryos were transformed with an expression vector pLem2VGM2 containing the *gastrodianin* gene (Ng *et al.*, 2007). Expression of *gastrodianin* was driven by a spike-specific Lem2 promoter (Abebe *et al.*, 2005). To assess ploidy, chromosome number was counted from root tips of transgenic seedlings. T_2 seeds were germinated in Petri dishes in the dark for 1 to 2 days. Root tips were pre-treated with saturated 1-bromonapthalene solution overnight at 4°C. Root tips were then fixed in 1:3 glacial acetic acid:95% ethanol solution at 4°C, hydrolyzed in 1M HCl at 60°C for 5 minutes, and stained in Fuelgen solution. The root tips were squashed on glass slides in a drop of 1% aceto-carmine and chromosomes were visualized under a microscope.

Greenhouse Screening of Transgenic Barley for FHB Resistance - Screening of transgenic Golden Promise barley plants for resistance to FHB was performed in a greenhouse at the University of Minnesota, St. Paul, MN. T_2 plants from seven events (event numbers 48, 50, 51, 52, 53, 56, and 58) were each grown in eight pots with five plants per pot. Non-transformed (wild-type) Golden Promise and transgenic Golden Promise expressing only *gfp* (Lem2Bgfp-GP) were included as negative controls. Conlon (FHB susceptible two-row), M122 (FHB moderately resistant six-row), Stander (moderately susceptible six-row), and Robust (FHB moderately susceptible six-row) were included as checks. Macroconidia of *F. graminearum* isolate Butte86ADA-11, cultured on mung bean agar (Evans *et al.*, 2000) were used as inoculum. Plants were spray inoculated at anthesis using 2 ml /head of a 1 x 10^5/ml macroconidial suspension, which was applied to both sides of the head with an airbrush sprayer. To facilitate infection, inoculated plants were kept at 100% humidity for 72 hours in a dew chamber. Following the incubation period plants were maintained in a greenhouse until assessed. FHB incidence and severity were assessed visually 14 days post-inoculation. FHB severity was calculated as the percentage of symptomatic spikelets/spike. The mean FHB severity for each transgenic line, Lem2Bgfp-GP (negative transformed control), and checks was compared with the wild type Golden Promise using Student's *t*-test.

RESULTS AND DISCUSSION

Phenotype of transgenic plants - We recovered fertile plants from 10 transformation events. Most had abnormal phenotypes including slow maturation, stunted growth, reduced seed set, twisted leaves, and bushy growth habit. Cytological analysis of T_2 plants revealed that transgenic plants with abnormal phenotypes had a tetraploid chromosome set (Table 1). Plants from event 58 had normal phenotypes with normal chromosome numbers. Observation of abnormal phenotypes is in agreement with many studies that used particle bombardment (Choi *et al.*, 2000; Filipecki and Melepszy, 2006). Particle bombardment often leads to complex patterns (multiple copies) of transgene integration (Filipecki and Malepszy, 2006). Moreover, the transgenes can disrupt endogenous genes, which can contribute to the development of strange phenotypes. Regeneration of plants from *in vitro* cultures exposes transformants to extra stresses due to selection agents (herbicide), osmotic effects from culture media, and insufficient nutrient supply or uptake (Latham *et al.*, 2006). These stress factors could lead to polyploidy, aneuploidy, chromosome rearrangements, somatic recombination, gene amplification, excision and insertion of retro-transposons, DNA methylation, and histone modifications (Filipecki and Malepszy, 2006). Mutations may also accumulate as time in tissue culture increases (Fukui, 1983). In our study, transgenic calli were maintained in tissue culture for 4 to 5 months, which may have increased somaclonal variation and contributed to the observed polyploidy.

Resistance of transgenic plants to FHB - T_2 plants from seven transformation events (two lines per event) expressing *gastrodianin* were evaluated for resistance to FHB (Table 2). Comparison of disease severity in transgenic plants expressing *gastrodianin*, the negative control (Lem2Bgfp-GP) and checks with the wild type Golden Promise indicated that the transgenic lines 50A4, 50D3, and 51E2 had significantly higher levels of FHB infection. Transgenic lines from event

Table 1. Chromosome number and phenotypes of transgenic barley plants expressing *gastrodianin*.

Transformation event	Transgenic lines	Plant[§]										No. of chromosomes
		1	2	3	4	5	6	7	8	9	10	
48	48C1	nh sh	nh sh tl	*	*	ns *	*	*	*	missing	missing	31 chromosomes
	48D1	hb *	ns *	ns tl	ns sh	*	ns *	*	missing	missing	missing	
50	50A4	*	*	*	*	*	*	*	*	missing	missing	
51	51E5	*	*	*	*	*	*			missing	missing	
52	52D6	*	*	*	*	*	nh sh	sh *	*	missing	missing	
	52G2	*	*	*	*	*	*	*		missing	missing	
53	53A1	ns sh tl	*	*	*	*	*	*	*	ns sh *	*	29 chromosomes
	53B1	sh *	sh *	sh *	ns sh *	missing	missing	missing	missing	missing	missing	
56	56A1	*	*	*	*	*	*	*	*	*	tl *	30 chromosomes
	56C1	*	*	*	*	*	*	*	*	*	missing	32 chromosomes
58	58B5	n	n	n	n	n	n	n	n	n	missing	Normal
	58D5	n	n	n	n	n	n	n	n	n	n	Normal

[§] hb, head branching; n, normal; nh, no head; ns, no seed; sh, short; tl, thin leaves; *, broad leaves, large seed, partial sterility, resembles tetraploid.

Table 2. FHB severity (% symptomatic spikelets/spike) for transgenic barley lines expressing *gastrodianin* and FHB checks. Values are means of 6–8 replications, with each replicate consisting of 3–5 plants.

Plants	FHB severity (%)
A. Check lines	
1. Non-transgenic	
Golden Promise	55.9
Conlon (FHB susceptible, 2-row)	89.0*
Robust (FHB moderately susceptible, 6-row)	69.7
Stander (FHB moderately susceptible, 6-row)	60.9
M122 (FHB moderately resistant, 6-row)	74.4
2. Transgenic (expressing gfp only)	
Lem2Bgfp-GP	43.5
B. Transgenic lines expressing gastrodianin	
48A1	77.7
48B3	72.6
50A4	81.5*
50D3	84.3*
51E2	80.3*
52D6	77.1
52G2	73.2
53A1	55.5
56A1	61.2
56D3	73.6
58B5	45.5
58D5	24.0*

**Indicates significantly different FHB severity ($p<0.05$) compared with non-transgenic Golden Promise.*

58 either had similar (58B5) or lower (58D5) FHB severity. Among the checks, only Conlon had significantly higher FHB infection than the wild-type Golden Promise (Table 2).

In our study, the average percentage of FHB infection varied from 24% (plant 58D5) to 84% (plant 50D3). One major difference between transgenic plants from event 58 and the susceptible transgenic plants was that the former had normal sets of chromosomes whereas the latter were tetraploids (Table 1). It is possible that abnormal chromosome numbers may have made plants more susceptible to FHB. Another reason why the response to FHB infection varied widely among the transgenic lines may be differences in the location of transgene insertions (position effect). Sequences surrounding the transgene will likely influence transgene expression and stability. Independent transgenic lines that contain the same copy number sometimes show differences in expression by as much as a 100-fold due to positional effects (Filipecki and Malepszy, 2006).

FUTURE PLANS

Our greenhouse evaluation of transgenic plants expressing *gastrodianin* has produced at least one line (58D5) that has improved resistance to FHB. However, the test needs to be repeated to make sure the response observed is real. We are currently repeating the greenhouse experiment. Field testing of transformants is the best way to accurately determine FHB resistance under natural conditions. Most of our transformants are tetraploids and, even with the diploid plants, crossing to an elite variety is necessary to remove any unwanted traits such as low seed

setting, stunted growth and slow maturity (Bregitzer *et al*., 2008). Dr. Lynn Dahleen has crossed selected transformants into Conlon (female parent). Field evaluation of the crosses is underway.

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ASSOCIATION ANALYSIS OF FHB RESISTANCE DERIVED FROM TUNISIAN 108 IN DURUM WHEAT

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ABSTRACT

Fusarium head blight (FHB) is a devastating disease of wheat (*Triticum aestivum* L.) world-wide, causing tremendous losses in grain yield and quality. The main species that causes FHB in the United States is *Fusarium graminearum* (Scab). This destructive fungal disease caused two billion dollars (direct revenue loss) in the period of 1993 to 2001 in the United States alone, while indirect loss estimated almost three times of this amount. Durum wheat has been heavily impacted, with a 44% loss of value in the U.S. crop, which is grown primarily in North Dakota. Thus, it is critical to identify means of defeating this disease or reducing its pathogenic effect to enhance wheat production.

In the previous report we used 171 BC_1F_6 and 169 BC_1F_7 lines derived from crossing of four Tunisian tetraploid sources of resistance (Tun7, Tun18, Tun34, Tun36) with durum cultivars 'Ben', 'Maier', 'Lebsock' and 'Mountrail' for association studies. The Tun18 and Tun7 expressed similar resistance level to FHB as compared with the best hexaploid wheat sources (i.e. Sumai3 and Wangshuibai). A new significant QTL for FHB resistance was identified on the long arm of chromosome 5B (*Qfhs.ndsu-5BL*) with association mapping analysis. Linkage disequilibrium (LD) blocks extending from 40 to70 cM were evident in these populations. The results of association mapping analysis also demonstrated a region on the short arm of chromosome 3B as potentially linked to FHB resistance. This region is in proximity of major FHB resistance gene "*fhb1*" reported in hexaploid wheat. This finding was surprising considering the distance and lack of relationship between Tunisian tetraploid sources studied here and Chinese sources used to identify *fhb1*.

In the current study, two additional Tunisian- derived advanced backcross populations, Tun 108 × Lebsock/Lebsock and Tun 108 × Ben/Ben, were screened for FHB resistance in both greenhouse and field. Although there are obvious discrepancies between the two set of data because of environmental effect, on the average, 53 out of 173 (30.64%) and 57 out of 170 (33.53%) lines showed less than 20% infection in Tun 108 × Lebsock/Lebsock and Tun 108 × Ben/Ben populations, respectively. Both populations were genotyped using DArT (Diversity Array Technology®) clones and resulted in 553 polymorphic loci that mapped on the A and B genomes. Preliminary pedigree based association analysis of QTL results on these populations will be presented and compared with our previous results on Tunisian-derived lines.

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INDUCTION OF PLANT DEFENCE GENE EXPRESSION BY ANTAGONISTIC LIPOPEPTIDES FROM *PAENIBACILLUS* SP. STRAIN B2

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ABSTRACT

With the aim of obtaining new strategies to control plant diseases, we investigated the ability of antagonistic lipopolypeptides (paenimyxin) from *Paenibacillus* sp. strain B2 to elicit hydrogen peroxide (H_2O_2) production and several defence-related genes in the model legume *Medicago truncatula*. For this purpose, *M. truncatula* cell suspensions were used and a pathosystem between *M. truncatula* and *Fusarium acuminatum* was established. In *M. truncatula* cell cultures, the induction of H_2O_2 reached a maximum 20 min after elicitation with paenimyxin, whereas concentrations higher than 20 μM inhibited H_2O_2 induction and this was correlated with a lethal effect. In plant roots incubated with different concentrations of paenimyxin for 24 h before inoculation with *F. acuminatum*, paenimyxin at low concentration (c.a. 1 μM) had a protective effect and suppressed 95% of the necrotic symptoms, whereas a concentration higher than 10 μM had an inhibitory effect on plant growth. Gene responses were quantified in *M. truncatula* by semi-quantitative reverse transcription–polymerase chain reaction (RT–PCR). Genes involved in the biosynthesis of phytoalexins (phenylalanine ammonia-lyase, chalcone synthase, chalcone reductase), antifungal activity (pathogenesis-related proteins, chitinase) or cell wall (invertase) were highly up-regulated in root or cells after paenimyxin treatment. The mechanisms potentially involved in plant protection are discussed.

IDENTIFYING AND CHARACTERIZING BARLEY GENES THAT PROTECT AGAINST TRICHOTHECENES

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ABSTRACT

Our overall goal is to identify genes that play a role in resistance to Fusarium Head Blight (FHB) and to develop and test transgenic wheat carrying these genes. In particular, we are interested in identifying genes that protect barley and wheat from the effects of trichothecenes. Previously, we conducted a large set of RNA profiling experiments during *Fusarium graminearum* infection of barley and inoculation with the trichothecene deoxynivalenol (DON). We identified a set of potential resistance genes that respond to trichothecene accumulation. The potential resistance genes encode a cysteine synthase, ABC transporters, UDP-glucosyltransferases, cytochrome P450s, and glutathione-S-transferases (GST). From our RNA profiling experiments, we identified ten barley UDP-glucosyltransferases and cloned eight full-length cDNAs for testing in yeast. We identified a barley UDP-glucosyltransferase gene that exhibits DON resistance based on the yeast assay. As proof of concept, we generated transgenic *Arabidopsis* overexpressing the barley UDP-glucosyltransferase and tested these plants for their ability to grow on media containing trichothecene mycotoxins such as deoxynivalenol (DON) and 4,15-diacetoxyscirpenol (DAS). After 4 weeks of growth on DON-containing media, the wild-type seedlings were albino and had ceased growing. Shoot and root growth were not inhibited in the UDP-glucosyltransferase overexpression lines grown on media containing 10, 15 and 20 ppm of DON. During DAS treatment, the seedlings of these overexpression lines showed an obvious difference for root length (longer) and general plant health compared with the control. These results showed that overexpression of UDP-glucosyltransferase in transgenic *Arabidopsis* protect plants from the deleterious effects of DON and DAS. We are developing transgenic wheat plants upregulating this UDP-glucosyltransferase gene. Currently, we have isolated three barley genes encoding GSTs and are developing transgenic *Arabidopsis* carrying these genes.

TRANSPOSONS BASED SATURATION MUTAGENESIS TO EXPLORE FHB RESISTANCE IN BARLEY

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ABSTRACT

Fusarium head blight is a devastating epidemic disease of wheat and barley that causes heavy economic losses to farmers due to yield decreases and production of mycotoxin that renders the grain useless for flour and malt products. Barley varieties resistant to FHB is a matter of high priority in many areas where they are grown, but the complex nature of resistance make this a highly challenging task. Two major QTL's have been identified viz. QTL1 and QTL2 on chromosome 6H and 2H respectively which have a large effect on kernel discoloration. The resistant allele of QTL2 decreases the occurrence of head blight by nearly 50% in varieties in which it is present thus proving its importance. Efforts have been made to clone important QTLs for better understanding of the mechanisms involved for FHB tolerance. Maize *Ac*/*Ds* system is one of the important tools that can be utilized for dissecting and saturating QTLs through saturation mutagenesis. Previous and ongoing mapping studies in our lab indicate an added advantage of *Ds* transpositions, in gene rich linked positions; making this technique appropriate to dissect FHB QTLs. Currently, our main focus is to saturate QTL2 region using maize *Ds* elements eventually facilitating identification and characterization of genes associated with FHB resistance. Plants with single *Ds* insertions (TNPs), mapping near QTLs of interest are important vehicles for gene identification through re-activation and transposition of *Ds*. *Ds* elements from TNP 41 (mapped near QTL2) were re-activated by crossing them with *Ac*TPase-expressing plants. In this population, we have identified some phenotypes, morphology of which may be associated with FHB tolerance. This effort of saturation mutagenesis with *Ds* transposons will lead to a better understanding of FHB resistance and candidate genes that display quantitative variation.

GENE EXPRESSION ANALYSIS OF RELATED WHEAT LINES WITH CONTRASTING LEVELS OF HEAD BLIGHT RESISTANCE AFTER *FUSARIUM GRAMINEARUM* INOCULATION

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ABSTRACT

Eight spring wheat genotypes with contrasting phenotypes for FHB resistance were used in this study: the highly resistant line CM82036, the highly susceptible cultivar Remus, four BC_5F_2 near isogenic lines (NILs) for *Fhb1* and *Qfhs.ifa-5A* and two doubled haploid (DH) lines from a CM82036/Remus mapping population differing in *Fhb1* and *Qfhs.ifa-5A*.

At anthesis the flowering ears of the plants were single floret inoculated by *F. graminearum* or water. The inoculated spikelets were harvested at several time points after inoculation and dissected into the generative and vegetative parts for RNA preparation. Differential gene expression was monitored with two complementary methods: 1) cDNA-AFLPs or 2) using the Affymetrix wheat GeneChip. At early time points (8-24 hpi) after inoculation only few genes were differentially expressed, at later time points (48-72 hpi) an increasing number of differentially expressed transcripts was evident. A comparative analysis of the data on identified candidate genes gained by the two complementary approaches will be presented.

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CHARACTERIZATION OF AN MRP INVOLVED IN THE WHEAT RESPONSE TO THE MYCOTOXIN DEOXYNIVALENOL

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ABSTRACT

Previously we identified several wheat genes that were responsive to the Fusarium mycotoxin deoxynivalenol (DON) and that were associated with the ability of the wheat cultivar CM82036 to resist the deleterious effects of this toxin. We have cloned and sequenced one such gene, namely a multidrug resistant protein (MRP) ABC transporter. Phylogenetic analysis indicated that it clusters with clade II and the MRP3 subfamily of MRP transporters. Gene expression studies indicated that it is more DON-up-regulated in cultivar CM82036 as compared to the DON-susceptible cultivar Remus. Additionally, it is up-regulated in response to jasmonic acid. The effect of DON on TaMRP3 transcript accumulation in wheat was more pronounced than that of the more potent protein synthesis inhibitor CHX, suggesting that its activation is not merely a secondary effect of toxin-mediated inhibition of protein synthesis. Ongoing work is determining the functional significance of the encoded protein in plant responses to xenobiotics.

UNRAVELLING THE WHEAT RESPONSE TO THE PROTEIN SYNTHESIS INHIBITOR DEOXYNIVALENOL

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ABSTRACT

Several wheat genes, including a MRP ABC transporter gene and two cytochrome P450 genes are responsive to the *Fusarium* mycotoxin deoxynivalenol (DON). We found that the accumulation of these transcripts in response to the *Fusarium* mycotoxin DON was significantly higher, and occurred earlier, in the DON-resistant cultivar CM82036 as compared to susceptible cultivar Remus, as revealed using gene expression studies. Based on the nature of these transcripts, insights are gained into how plants respond to, transform, and resist the harmful effects of, the toxin. Analysis of the effect of DON on the transcriptome of cultivar Remus yielded further insights into how a susceptible host responds to DON. The results support the theory that ubiquitin-proteasome system components play an important role in the plant response to DON. Furthermore, they provide evidence that jasmonates and phenylpropanoids contribute to the host response to this toxin.

ASSOCIATION STUDIES VALIDATE AND DISCOVER GENETIC LOCI FOR WHEAT FUSARIUM HEAD BLIGHT RESISTANCE

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ABSTRACT

Wheat Fusarium head blight (FHB) is a destructive wheat disease worldwide. To validate and identify quantitative trait loci (QTL) for wheat FHB resistance (type II), association study was conducted using a collection of 149 Asian wheat accessions and 205 U.S. hard and soft winter wheat breeding lines. FHB was evaluated for three greenhouse seasons from 2008-2010 by injecting ~1000 conidiospores into the central spikelet of a spike and measuring the proportion of symptomatic spikelets (PSS) at 16 day after inoculation (DAI) at Kansas State University. In general, Asian accessions had a relatively higher type II resistance than that of U.S. accessions. A total of 282 SSR markers covering all wheat chromosomes including those linked to known QTL for FHB resistance were used to genotype the population. Statistical model tests selected the unified linear mixed model (ULMM) for association computation. Eighteen marker alleles showed significant association with FHB resistance in Asian population including three previously reported QTLs on 3BS, 3BSc, and 5AS. Four marker alleles for 5AS QTL linked to FHB susceptibility in the Asian group suggested most of Asian accessions in this study may lack the resistance allele on chromosome 5AS. Marker *Xgwm276* on 7A was significantly associated with FHB resistance in the Asian group, which has not been reported previously. Twelve accessions with the *Xgwm276*-110 allele had a mean PSS of 0.14 that is lower than these accessions with marker allele *Xgwm533*-159 (PSS= 0.21) on 3BS. In the U.S. population, 18 alleles from 17 markers were significant associated with FHB resistance. Two previously reported QTLs on 3BS (*Xgwm493* and *Xbac102*) and 4D (*Xbarc98*, *Xwmc473*, and *Xgwm608*) were validated. Among all 17 significant markers, two novel marker alleles, *Xcfa2263*-140 (2A) and *Xgwm320* -274 (2D), showed the largest effect on FHB resistance in the U.S. population with a mean of PSS of 0.38. Therefore, the QTL on 2A and 2D are likely new QTL for FHB resistance in U.S. accessions. The results not only validated previously reported important QTL, but also discovered some new QTL in germplasm from both Asian and the U.S. wheat.

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