GENETIC ENGINEERING

Chairperson: Kay Walker-Simmons

WHEAT TRANSFORMATION: A NEEDED TOOL FOR WHEAT GENETICS AND GERMPLASM IMPROVEMENT

P. S. Baenziger[1*], M. Dickman[2], T. Clemente[1], S. Sato[1], A. Mitra[2], J. Watkins[2], B. Langston[2], B. LaVallee[1], J. Schimelfenig[2], S. Mitra[1], J. Bohlmann[1] and M. Montgomery[1]

[1]Department of Agronomy and Horticulture; and [2]Department of Plant Pathology, University of Nebraska at Lincoln (UNL), Lincoln, NE, 68503-0915, USA
Corresponding Author: PH: (402) 472-1538; E-mail: agro104@unlnotes.unl

ABSTRACT

Fusarium head blight (FHB) is a major disease of wheat in the north central and eastern United States that affects both grain yield and end-use quality. It is generally agreed that the most cost effective way of preventing this disease would be through the release of FHB resistant cultivars. A major limitation to developing FHB resistant cultivars is the limited availability of resistant germplasm. Our project has attempted to increase genetic resources expressing FHB resistance through wheat transformation. Though microprojectile bombardment is commonly used, wheat transformation using *Agrobacterium tumefaciens* is preferred because a higher probability of simple, low copy integration events and ease in generating 'marker-free' transgenic lines by simultaneous delivery of two T-DNA elements. Currently we are using two strategies for increasing FHB resistance. The first strategy relies upon anti-fungal protein expression, such as bovine lactoferrin along with its derivative bovine lactoferricin, and the synthetic lytic peptide D4E1. The second strategy is to develop lines expressing inhibitors of programmed cell death (anti-apoptotic genes; Bcl-xL, Op-IAP, Sf-IAP, and ced9). FHB and its toxin, deoxynivalenol, have been suggested to regulate programmed cell death during pathogen infection. By transforming wheat with inhibitors of programmed cell death we hope to not only increase tolerance to FHB in wheat, but inhibit necrotrophic pathogen infection in general. The expression of inhibitors of programmed cell death may result in other agronomically beneficial traits as well. Preliminary results suggest improved tolerance to high levels of salinity and cold stress, as transgenic plants demonstrate reduced levels of DNA fragmentation and increased hardiness, respectively. We have also demonstrated the ability of deoxynivalenol to induce increased levels of programmed cell death via TUNEL staining of treated leaf sections. Finally, while our interest lays in the augmentation of FHB resistance present in wheat and its relatives, the importance of wheat transformation as a key genomics tools is clear. Transformation technology provides the ability to insert beneficial genes as well as silence existing genes in order to elucidate host-pathogen interactions during necrotrophic infection. For example, a newly discovered extremely efficient gene silencing system called DRIGS (direct repeat induced post transcriptional gene silencing) is currently being tested in wheat.

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MAPPING FHB RESISTANCE QTL IN A BARLEY POPULATION DERIVED FROM AN ATAHUALPA X M81 CROSS

K.A. Beaubien[1], L.M. Nduulu[1] and K.P. Smith[1*]

[1]Dept. of Agronomy and Plant Genetics, University of Minnesota, St. Paul, MN, USA
[*]Corresponding Author: PH: (612) 624-1211; E-mail: smith376@umn.edu

ABSTRACT

Previous barley mapping studies, using four sources of resistance (Chevron, Frederickson, Gobernadora and Zhedar), report four major *Fusarium* head blight (FHB) quantitative trait loci (QTLs): one on chromosome 2 near the centromere, one on chromosome 2 near the *Vrs1* locus, one on chromosome 2 near the southern telomere and one on chromosome 6. The first objective of this study was to identify QTL for resistance to FHB in an Atahualpa x M81 (AxM81) population in hopes that they will complement those previously identified. Atahualpa was chosen because it is genetically dissimilar to other sources of resistance in barley. The second objective was to investigate the effect of major spike morphology traits on the detection of FHB QTLs. To accomplish this, we created a selected subset of the AxM81 population that was fixed for two single gene traits that have a phenotypic association with FHB. The *nud1* locus, located on chromosome 1, determines covered v. hulless kernels. The *Vrs1* locus, located on chromosome 2, determines two-rowed v. six-rowed spike type. The random subset contains 102 individuals segregating at both the *nud1* and *Vrs1* loci. The selected subset contains 67 individuals fixed at both the *nud1* (for covered kernels) and *Vrs1* (for six-rowed spike type) loci. Phenotypic data for FHB severity was collected from four environments; deoxynivalenol (DON) accumulation data was collected from one environment. A simple sequence repeat (SSR) linkage map was constructed using JoinMap 3.0. The map currently covers approximately 60% of the barley genome. Composite interval mapping QTL analysis with PlabQTL 3.0 has located a major FHB QTL using the random population on chromosome 2 associated with the *Vrs1* locus at Crookston 2003 (LOD=13.9; R^2=46.9%), China 2003 (LOD=19.6; R^2=59.1%) and China 2004 (LOD=9.6; R^2=35.5%). A single DON QTL located in the random population was coincident with the major *Vrs1* QTL at Crookston 2002 (LOD=3.7; R^2=15.4%). One FHB QTL, identified in a single environment, was located in the random population on chromosome 2 associated with *GBM1062* (approximately 15 cM distal to *Vrs1*) at Crookston 2002 (LOD=3.3; R^2=14.1%). Three FHB QTLs, identified in single environments, were located using the selected population; two are located on chromosome 1 associated with *HvCMA* at China 2004 (LOD=3.2; R^2=20.2%), and *Bmag0321* at Crookston 2003 (LOD=3.1; R^2=19.5%), and one is located on chromosome 2 associated with *Bmac0093* at Crookston 2003 (LOD=4.0; R^2=23.9%). One DON QTL was located in the selected population on chromosome 1 associated with *Bmag0321* at Crookston 2002 (LOD=3.7; R^2=22.9%). Preliminary results indicate several QTL for FHB resistance were identified using the selected subsets that were not identified using the random subset.

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ROLE OF DIOXYGENASES IN FUNGAL SPORULATION

Marion Brodhagen, Dimitrios Tsitsigiannis and Nancy Keller*

Department of Plant Pathology, University of Wisconsin, Madison, WI, 53706, USA
*Corresponding Author: PH: 608-262-9795; E-mail: Npk@plantpath.wisc.edu

ABSTRACT

Oxylipins comprise a family of structurally related oxygenated long chain fatty acid-derived molecules that exhibit crucial biological activities as signals of intra- and inter-cellular communication in mammals, plants and fungi. Oxylipin production is ubiquitous among pathogenic and saprophytic fungi and appears to play a role in life cycle control particularly in sexual and asexual development. For instance, in various members of Mucorales, immunofluorescence microscopy showed that 3-OH oxylipins are associated with asexual reproductive structures (e.g. sporangium, columella and aggregating sporangiospores), and in the yeast *Dipodascopsis uninucleata* with the sexual reproductive phase of the life cycle (e.g. gametangia, asci and matrix of released aggregating ascospores). We have recently identified three fatty acid oxygenases (PpoA, PpoB and PpoC) in the model fungus *Aspergillus nidulans*. Deletion of the encoding genes were correlated with changes in the asexual to sexual spore development, alterations in mycotoxin biosynthesis and decreased virulence as measured by spore reduction on host seed. Phylogenetic analyses showed that *ppo* genes are present in both saprophytic and pathogenic Ascomycetes and Basidiomycetes, suggesting a conserved role for Ppo enzymes in the life cycle of fungi. We have identified four putative Ppo proteins in *Fusarium graminearum* and will describe strategies in inactivating these genes.

SATURATION MAPPING OF THE FHB RESISTANCE QTL *QFHS-NDSU-3A* IN TETRAPLOID WHEAT

X. Chen[1], J. Hu[2], S. Kianian[1] and X. Cai[1*]

[1]Dept. of Plant Sciences, North Dakota State University, Fargo, ND 58105; and
[2]USDA-ARS, Northern Crop Science Lab, Fargo, ND 58105
[*]Corresponding Author: PH: (701) 231-7404; E-mail: Xiwen.Cai@ndsu.nodak.edu

ABSTRACT

Fusarium head blight (FHB), a destructive disease of wheat, has posed a significant threat to wheat production, processing, and consumption. Sources of effective resistance to FHB have not been found in durum wheat (*Triticum turgidum* var. *durum* L., 2n=4x=28, AABB). A major FHB resistance quantitative trait locus (QTL) *Qfhs.ndsu-3AS* was identified from a wild tetraploid wheat accession (*T. dococcoides* L., 2n=4x=28, AABB) and mapped within a 29.3 cM interval on chromosome 3A. A mapping population of 83 recombinant inbred chromosome lines (RICLs) derived from a cross between the *T. turgidum* var. *durum* cv. Langdon (LDN)-*T. dicoccoides* substitution line 3A and LDN has been used for saturation mapping of this QTL region in the present study. To date, we have assigned 30 new molecular markers to the QTL region, which extended the map distance from 155.2 cM to 248.4 cM. These markers, including SSR, STS, TRAP (target region amplification polymorphism), SSCP (single-strand conformation polymorphism), and CAPS (cleaved amplified polymorphic sequence), were generated from the ESTs mapped within the deletion bin 3AS-4 where the microsatellite marker closely linked with the peak of the QTL, *Xgwm2*, was assigned. We have identified new markers flanking the QTL and placed the QTL within a 9.4 cM chromosomal interval that is over three times smaller than the previous interval (29.3 cM). Thermal asymmetric interlaced PCR (TAIL-PCR) has been employed to extend DNA sequences surrounding the loci of interest. Single or low-copy TAIL-PCR products have been used to screen BAC libraries of LDN and *T. tauschii* (2n=2x=14, DD) and generate more markers to saturate this QTL region. A large F_2 population (over 1,000 individuals) was developed from a cross between LDN and a RICL with a smaller *T. dicoccoides* chromosomal fragment containing *Qfhs.ndsu-3AS*. This population has been used to generate more recombinants for fine mapping of the QTL region. F_3 offspring of the heterozygous recombinant F_2 individuals were produced to generate homozygous recombinants for FHB evaluation. Comparative mapping suggested that the FHB resistance QTL *Qfhs.ndsu-3AS* and *Qfhs.ndsu-3BS* localized on the short arm of chromosome 3A and 3B respectively, are not homoeoloci.

TISSUE SPECIFIC EXPRESSION OF A CHITINASE GENE FROM *TRICHODERMA ATROVIRIDE* CONFERS *FUSARIUM* RESISTANCE TO GM-BARLEY

J.L. Clarke, S.S. Klemsdal* and O. Elen

Norwegian Crop Research Institute, Plant Protection Centre, Norway
*Corresponding Author: PH: (47) 64949400; E-mail: sonja.klemsdal@planteforsk.no

ABSTRACT

Since many *Fusarium*-mycotoxins are heat stable, these compounds cannot be removed through the chain of food processing, and once present in the grains at harvest, they will also be present in the final product. The reduction of the original infection of *Fusarium* will thus be the only way to reduce the amount of *Fusarium*-produced mycotoxins in the final food products. Cereal genes conferring resistance to *Fusarium* infection have not yet been identified, but in some wheat cultivars, resistance loci have been mapped. *Trichoderma* genes encoding chitindegrading enzymes have been introduced into several plant species and have been shown to increase the plants' resistance against fungal pathogens. At the Norwegian Crop Research Institute we have produced GM-barley where a fungal endochitinase gene, *ech42* from *T. atroviride* regulated by the barley promoter *Ltp2*, has been inserted resulting in increased resistance towards *Fusarium* infection of the seeds. The advantage of the *Ltp*2 promoter is that it permits a gene to be expressed only in the aleurone layer of developing seeds, corresponding to the point of time when *Fusarium* infects the spikes of barley. One of the resulting transformed plant lines, PL9, seemed to be especially promising. The copy number was estimated by the real-time PCR method to be low. Study on the inheritance of the transgenes in T_1 progeny revealed a 3:1 segregation. The expression of the chitinase gene, *ech42,* was studied in the T_1 generation using quantitative real-time RT-PCR assay. Some T_1 progenies showed very high *ech42* expression while others had either very low or no detectable expression. After inoculation with *Fusarium culmorum,* all *ech42* containing T_1 progenies coming from PL9 showed high resistance. The amount of *F. culmorum* present after point inoculation of the spikes was quantified by real-time PCR analysis. Extremely low amounts or no *F. culmorum* could be detected in seeds located at the same spike close to the point inoculated grains compared to the huge amounts found in wild type control plants. Further studies will be performed on plants from the T_2 generation currently grown in the greenhouse.

A TRUNCATED FORM OF RIBOSOMAL PROTEIN L3 ELIMINATES RIBOSOME DEPURINATION AND CELL DEATH CAUSED BY POKEWEED ANTIVIRAL PROTEIN AND CONFERS RESISTANCE TO TRICHOTHECENE MYCOTOXINS

Rong Di and Nilgun E. Tumer*

Biotechnology Center for Agriculture and the Environment, and the Department of Plant Biology and Pathology, Cook College, Rutgers University, New Brunswick, New Jersey 08901-8520, USA
*Corresponding Author: PH: 732-932-8165 x215; E-mail: tumer@aesop.rutgers.edu

ABSTRACT

The contamination of important agricultural products, such as wheat, barley or maize with the trichothecene mycotoxin, deoxynivalenol (DON) due to infection with *Fusarium graminearum* or *Fusarium culmorum* is a worldwide problem. Trichothecenes inhibit translation by targeting ribosomal protein L3. We have previously shown that pokeweed antiviral protein (PAP), a single chain ribosome inactivating protein, depurinates ribosomes by binding to L3. Co-expression of a truncated form of yeast L3 (L3D), which contains only the first 100 amino acids, together with wild type PAP in transgenic tobacco plants led to a dramatic increase in PAP mRNA and protein expression. Unlike plants expressing PAP alone, transgenic plants expressing wild type PAP and yeast L3D were phenotypically normal. Ribosomes from these plants were not depurinated, even though high levels of PAP was associated with ribosomes. Expression of the endogenous tobacco ribosomal protein L3 was upregulated in transgenic lines containing L3D and PAP. Transgenic lines that showed high level of PAP and L3 protein expression were resistant to the *Fusarium* mycotoxins, DON and 4,15-diacetoxyscirpenol (DAS). These results demonstrate that co-expression of yeast L3D and PAP eliminates ribosome depurination, mRNA destabilization and cell death caused by PAP and increases endogenous L3 expression. High levels of PAP and L3 expressed in these plants confer resistance to trichothecene mycotoxins.

OVEREXPRESSION OF ANTIFUNGAL PROTEINS INCREASES THE RESISTANCE OF WHEAT TO FUSARIUM HEAD BLIGHT

C.A. Mackintosh[1], J.M. Lewis[1], S.J. Heinen[1], L.E. Radmer[1], R. Dill-Macky[2], C.K. Evans[2], G.D. Baldridge[2], R.J. Zeyen[2] and G. J. Muehlbauer[1*]

[1]Department of Agronomy and Plant Genetics, 411 Borlaug Hall; and [2]Department of Plant Pathology, 495 Borlaug Hall, 1991 Upper Buford Circle, St Paul, MN 55108, USA
[*]Corresponding Author: PH: 612-625-6228; E-mail: muehl003@umn.edu

ABSTRACT

We are developing and testing transgenic wheat for resistance to Fusarium Head Blight (FHB). Anti-fungal proteins (AFPs) such as ß-1,3-glucanases, thionins, chitinases, thaumatin-like proteins (tlps) and ribosome-inactivating proteins (RIPs) are thought to inhibit fungal growth via different mechanisms. Chitinases and ß-1,3-glucanases degrade fungal cell walls, tlps and thionins degrade fungal cell membranes and RIPs inhibit fungal protein synthesis. Transgenic wheat lines over-expressing these AFPs in the cultivar Bobwhite were generated using micro-projectile bombardment. In transgenic lines carrying a ß-1,3-glucanase, an α-purothionin and a tlp-1, our previous results showed statistically significant reductions in scab severity compared to the nontransgenic Bobwhite controls in the greenhouse. These lines were evaluated in field trials in the summer of 2004 and five lines exhibited statistically significant reductions in scab severity compared to nontransgenic Bobwhite controls. We also developed seventeen and eight lines carrying a barley chitinase and barley RIP, respectively. In addition, we developed four, eleven and six lines expressing a combination of chitinase/RIP, chitinase/tlp-1 and RIP/tlp-1, respectively. These combinations each employ two of the three different mechanisms of fungal growth inhibition. We screened these lines for FHB resistance in the greenhouse three to four times. Eight chitinase, one RIP, three chitinase/tlp-1, one chitinase/RIP and three RIP/tlp-1 lines consistently show enhanced resistance towards FHB when compared to Bobwhite, the untransformed control. Western blot analyses of these lines are discussed.

EXPRESSION OF ARABIDOPSIS NPR1 IN WHEAT CONFERS RESISTANCE TO FUSARIUM HEAD BLIGHT

Ragiba Makandar[1], Harold N. Trick[2] and Jyoti Shah[1*]

[1]Division of Biology; and [2]Department of Plant Pathology,
Kansas State University, Manhattan, KS 66506 USA
[*]Corresponding Author: PH: 785-532-6360; E-mail: shah@ksu.edu;

ABSTRACT

Plant productivity and quality is severely limited by Fusarium Head Blight (FHB) or scab, which has re-emerged as a devastating disease of wheat and barley. Breeding has been at the forefront in developing wheat with improved FHB resistance. Biotechnology provides an alternative approach for augmenting resistance to FHB. The *NPR1* gene is a key regulator of basal and inducible defense responses in Arabidopsis to several pathogens. Moreover, over expression of NPR1 in Arabidopsis and rice was found to confer resistance to biotrophic pathogens. However, the impact of NPR1 on resistance to necrotrophs remains to be determined. We provide evidence that expression of NPR1 confers resistance to FHB in wheat. The Arabidopsis NPR1 gene was expressed in wheat plants from the ubiquitously expressed maize *Ubi1* promoter. A strong type II resistance to FHB was observed in two independently derived *Ubi1::AtNPR1* transgenic lines. FHB resistance was inherited as a dominant trait; significant correlation was observed between the FHB resistance phenotype and the expression of the *Ubi1*::*AtNPR1* transgene. Expression of the transgene and FHB resistance was stably maintained in the T_2 and T_3 generations. Comparisons of grain yield between a transgenic line and the non-transgenic control plant revealed no detrimental effects of the *Ubi1::AtNPR1* transgene expression on grain yield in healthy green house grown plants. These two promising lines are being readied for field studies on yield, the durability of FHB resistance and broad-spectrum resistance to other pathogens.

Previously, we had cloned a partial cDNA for a wheat homolog (*WhNPR1*) of the Arabidopsis and rice *NPR1* genes from a rust-infected Lr21 wheat cDNA library. The predicted WhNPR1 protein exhibits 80% similarity to the Rice NPR1 protein. We have used RACE to clone the 5‘end of the *WhNPR1* gene. Using this RACE product, we are currently reconstructing the full-length *WhNPR1* cDNA. Since NPR1 function in plant defense requires its interaction with other proteins, we hypothesize that increased expression of WhNPR1 will be more effective than the Arabidopsis NPR1 in conferring durable resistance to FHB in wheat. To test the hypothesis, we will generate transgenic plants that overexpress WhNPR1.

MICROARRAY ANALYSIS OF BARLEY INFECTED WITH *FUSARIUM GRAMINEARUM*

Gary J. Muehlbauer[1*], Jayanand Boddu[1], Warren Kruger[1] and Seungho Cho[1]

[1]Department of Agronomy and Plant Genetics, University of Minnesota, St. Paul, MN 55108, USA
[*]Corresponding Author: PH: (612) 625-6228; E-mail: muehl003@umn.edu

ABSTRACT

Fusarium head blight (FHB), caused by *Fusarium graminearum*, is a serious problem for barley and wheat cultivation. The objectives of this study were to identify barley defense response mechanisms operating against *F. graminearum*. The Barley1 Affymetrix GeneChip provides a means for evaluating the differential transcript accumulation in barley from large sets of genes under defined conditions. We used the Barley1 GeneChip to study the differential transcript accumulation from barley genes in spikes challenged with *F. graminierum* and mock inoculation water controls. We also examined *F. graminearum* infection structures and deoxynivalenol (DON) accumulation. Four replicate experiments were conducted at five different time points, 24h, 48h, 72h, 96h and 144h. Two classes of genes were identified namely, quantitatively expressing and qualitatively expressing genes. Genes exhibiting quantitative differences in transcript accumulation were defined as those transcripts that accumulated in the *Fusarium*-treated spikes at a statistically significant higher level than the water controls. A total of 186 such genes were found. Genes exhibiting qualitative differences in transcript accumulation are those that are exclusively found in the water controls or the *Fusarium* treated samples. A total of 389 such genes were found. Twenty genes were randomly selected and validated on the northern blots, indicating the GeneChip data are robust. Based on the defined patterns of differential transcript accumulation, histology and DON accumulation, we classified the disease progression into four broad classifications: preinfection (24h), early (48h), middle (72h and 96h) and late phase (144h). Functional classification of the identified genes was done based on annotation, number and mean expression values and attempts were made to tag the biological significance to these groups. One major pathway showing significant induction during *Fusarium* infection was found to be tryptophan metabolism. Our results show the power of the Barley1 GeneChip to identify patterns and pathways of genes expression during *F. graminearum* infection.

HIGH RESOLUTION MAPPING OF FUSARIUM HEAD BLIGHT RESISTANCE AND HEADING DATE QTL ON CHROMOSOME 2H OF BARLEY

Lexingtons M. Nduulu, A. Mesfin, G.J. Muehlbauer and K.P. Smith*

Dept. of Agronomy and Plant Genetics, University of Minnesota, St. Paul, MN 55108, USA
*Corresponding Author: PH: (612) 624-1211; E-mail: smith376@tc.umn.edu.

OBJECTIVES

To identify precise locations of Fusarium head blight resistance and heading date QTL on chromosome 2H and determine if the association between the two traits is due to linkage or pleiotropy.

INTRODUCTION

A major QTL for Fusarium head blight (FHB) resistance, discovered in the Chevron x M69 mapping population was located in a 45 centimorgan (cM) genomic region of chromosome 2 (2H) (de la Pena et al., 1999). The resistant allele at this QTL was also associated with late heading. A follow-up validation study comparing the Chevron x M69 mapping population with two other Chevron derived populations confirmed the coincidence of HD and FHB QTL in this region (Canci et al., 2004). Subsequently, a marker-assisted selection (MAS) study using markers carrying the Chevron allele at the chromosome 2H QTL region resulted in a 43% reduction in FHB severity and a 2-day increase in heading date (HD) (Gustus et al., 2001). In a more recent study, six backcross (BC3) near-isogenic lines carrying the chevron alleles for markers in the chromosome 2 FHB QTL region were found to reduce FHB by 44% in St. Paul and 41% in Crookston (Nduulu et al., 2002). This same QTL region also increased HD by six days; thus creating uncertainty as to whether the association is due to linkage or pleiotropy.

In this current study, we report the construction of a fine map for the chromosome 2H target QTL region using an F2 population derived from a cross between a BC5 line carrying the Chevron alleles in the QTL region and the recurrent parent M69. Because these backcross-derived F2 lines are isogenic for the entire genome and only segregate at the target QTL region, evaluating the recombinant lines allows us to more precisely estimate QTL positions for FHB and HD.

MATERIALS AND METHODS

Development of the parental near isogenic line (pNIL): To develop the parental NIL, a progeny from the 101 F4:7 mapping population (de La Pena et al., 1999) was crossed with an elite line M69. Subsequently, a marker-assisted backcrossing scheme using M69 as the recurrent parent was used to advance lines to the BC4F2 generation. A BC4F2 line carrying the FHB-resistance Chevron alleles at the target QTL region was selected as the pNIL.

Development of recombinant NILs (rNILs): We derived an F2 population of 532 plants from a cross between the pNIL and M69. The F2s were screened with SSR markers *Bmag0140* and *Ebmac0521* flanking the target QTL region and 40 rNILs were identified that had a recombination event between the flanking markers. These 40 putative recombinants were further screened with 11 additional SSR markers previously mapped at the *Bmag0140-EBmac0521* interval. Using marker data from the entire F2 population, a linkage map for the target QTL region was created using the GMendel 3.0 program (Holloway and Knapp, 1994). The 40 rNILs were further advanced to the F2:4 generation and used for field testing.

Field evaluations of rNILs: The rNILs and the parental lines Chevron, M69, and pNIL were evaluated at St. Paul and Crookston, MN, in the summer of 2003 and 2004. The experimental design at each environment was a randomized complete block design with 3 replications. Entries were planted in 2.4 m

long single-row plots, spaced at 30 cm apart. At St. Paul, a macroconidia inoculation technique was used whereas at Crookston a grain-spawn inoculation technique was used (Mesfin et al., 2003). Nurseries were mist-irrigated daily to enhance disease. Entries were scored for % FHB severity by examining 20 random spikes from each plot and the number of infected spikelets from each spike counted and expressed as a percent of the total spikelets present. Heading date was scored as the number of days after planting to 50% emergence from the boot.

Statistical Analysis: The genotype x environment interaction effects were determined using Proc GLM (SAS Institute, 2000). The analysis revealed a significant G x E effect for both HD and FHB among rNILs. Further analyses were, therefore, performed on a per environment basis. Trait means for parental lines and rNILs were compared using protected LSD. The association between specific markers in the target QTL region and measured traits was determined by simple interval mapping (SIM) using PlabQTL (Utz and Melchinger, 1996).

RESULTS AND DISCUSSION

The rNILs carrying the Chevron allele at different segments at the chromosome 2H QTL region differed significantly for FHB and HD, indicating that the QTL for HD and FHB resistance were segregating amongst the rNILs. The pNIL used to develop the fine mapping population did not differ from the Chevron for FHB severity, but was slightly earlier than Chevron for HD (Table 1). This suggests the QTL for FHB on chromosome 2H is responsible for most of the resistance from Chevron. However, additional alleles at loci for HD outside the chromosome 2H target region likely contribute to the late heading of Chevron.

A total of 13 SSR markers, 9 from the linkage map of Canci et al., (2004), and four additional markers previously mapped in the same region by Ramsay et al., (2000) were genotyped for fine mapping (Fig. 1). Of these, *EBmac0849* mapped in the same location as *Bmac0093* and was subsequently dropped from the analysis. For the most part, the marker order was consistent with the original map. The total distance covered by the new map is 17.4 cM compared with the 44.9 cM distance covered by the updated Chevron x M69 map of Canci et al., 2004.

A major QTL for HD was detected between markers *HVBKASI* and *Bmag0015* (1 cM apart) at all the four environments tested and explained 40-80% of the phenotypic variation (Fig. 1; Table 2). A separate QTL for FHB flanked by markers *Bmag0140* and *Bmac0132* (3.5 cM apart) was detected 2 cM away from the HD QTL. This FHB QTL was detected in 2 of the 4 environments tested and explained 40-50% of the variance. Failure to detect FHB QTL in all environments was most likely due to poor disease levels experienced in the environments where QTL were not detected (Table 1). In conclusion, these data indicate that the association between FHB and HD is due to linkage (2 cM apart) rather than pleiotropy.

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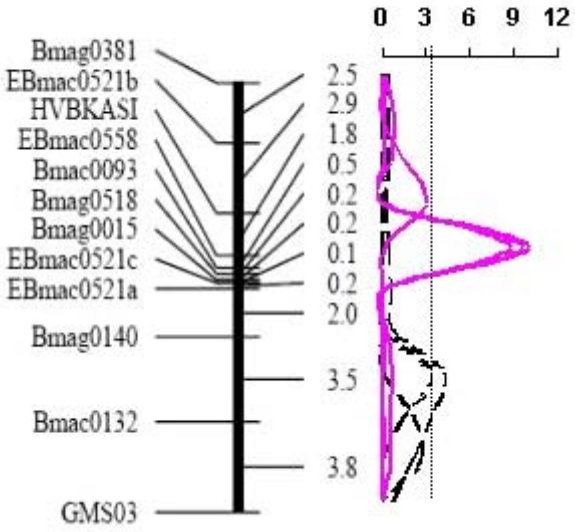

Figure 1. SSR fine map of the chromosome 2H FHB target QTL region and LOD score scan for the QTL associated with Fusarium head blight (FHB) severity and heading date (HD). Scans are shown for St. Paul 2003 (Sp03), Crookston 2003 (Cr03), St. Paul 2004 (Sp04) and Crookston 2004 (Cr04). Dark scan line is for FHB and dotted scan line is for HD.

Table 1. Means of the parents (pNIL, Chevron and M69) and recombinant near isogenic lines (rNILs) for percent Fusarium head blight severity (FHB) and heading date (HD).

Trait	Environment	pNIL	Chevron	M69	rNIL	
					Mean	Range
FHB	Sp2003	1.8a	1.3a	3.7b	2.4	1.0-4.4
	Cr2003	2.3a	3.8a	7.2b	5.5	0.7-13.1
	Sp2004	11.3a	8.5a	12.3a	9.4	1.9-20.6
	Cr2004	6.6a	1.6a	21.5b	17.6	4.6-59.7
HD	Sp2003	58.5b	60.0c	55.0a	58.0	54.0-61.0
	Cr2003	58.5b	61.0c	56.5a	58.1	54.0-61.0
	Sp2004	55.0b	55.7b	49.3a	56.7	48.3-57.0
	Cr2004	66.7b	68.7c	64.3a	66.0	62.3-68.7

Means within the same row followed by the same letter are not significantly different (P?0.05). Sp2003 = St. Paul, MN, 2003; Cr2003 = Crookston, MN, 2003; Sp2004 = St. Paul, MN, 2004; Cr2004 = Crookston, MN, 2004.

Table 2. Significant QTL (LOD>3.0) associated with Fusarium head blight (FHB) severity and heading date (HD) at four environments in the Chevron/M69 fine mapping population of recombinant near isogenic lines.

Trait/ Pos[1]	Marker interval	St. Paul 2003			Crookston 2003			St. Paul 2004			Crookston 2004		
		LOD	%Exp[2]	Add[3]	LOD	%Exp	Add	LOD	%Exp	Add	LOD	%Exp	Add
FHB													
11,13	Bmag0140-Bmac132				3.43	43.2	-2.82				4.21	50.0	-14.37
HD													
6	HVBKASI-EBmac0558										3.05	44.3	1.63
7	Bmag0518-Bmag0015	8.74	76.3	2.59	10.02	80.8	2.75	9.62	79.5	3.47			

[1]Pos = centimorgan position.
[2]% Exp = Percent phenotypic variance explained by QTL.
[3]Add = Additive effect of the Chevron allele on FHB severity and heading date expressed as regression coefficient.

A GFP REPORTER STRAIN FOR MONITORING TRI5 GENE ACTIVITY IN *FUSARIUM GRAMINAERUM*

L. Niessen*, G. Kalinowski, S. Theisen and R.F. Vogel

Technische Universität München, Lehrstuhl für Technische Mikrobiologie,
Weihenstephaner Steig 16, D-85350 Freising, Germany
*Corresponding Author: PH: 49 8161 715496; E-mail: niessen@wzw.tum.de

ABSTRACT

Fusarium graminearum is the most serious pathogen within the Fusarium Head Blight complex of fungal species. It produces the trichothecene mycotoxin Deoxynivalenol (DON) as a major virulence factor in the host-pathogen interaction. The TRI5 gene has a key role in the biosynthesis of trichothecene mycotoxins. We have developed a reporter system by transformation of *F. graminearum* TMW 4.0122 with the eGFP gene under control of the TRI5 promotor. A 926 bp fragment upstream of the TRI5 start codon containing the promotor region as well as a 342 bp portion upstream from the 3´end of the TRI5 coding region of a single spore isolate of *F. graminearum* TMW 4.0122 were cloned in *E. coli* DH5±. Fragments were excised and ligated via *Hin*dIII restriction sites newly introduced by modified primers. The ligation product was cloned into the pSM2 vector via *Pst*I and *Cla*I restriction sites to result in transformation vector pSM2GK1871, which was linearized by restriction of a singular *Hin*dIII site. Protoplasts of *F. graminearum* TMW 4.0122 were obtained by treatment of germinated conidia with driselase (Interspex) at 30 °C for 3 h. Protoplasts were separated and transformed with linearized pSM2GK1871. Selection on hygromycin B agar (150 µg/ml) revealed 88 transformants. Clones were subcultured on GYEP agar plates and inspected for expression of eGFP under the fluorescence microscope (Olympus). One clone (10/2/1) displayed intense green fluorescence emission at 510-550 nm upon excitation at 470-490 nm after 15 d of incubation at 25 °C. No such fluorescence was seen in the wild type strain grown under the same conditions. Fluorescence in the transformant was limited to a specific type of cells, which showed a characteristic yellow pigmentation when inspected under the light microscope. Such cells were also present in the wild type mycelium, with no green fluorescence emitted upon excitation at 470-490 nm. We are currently investigating whether trichothecene production in *F. graminearum* TMW 4.0122 might be restricted to specialized cells ("toxocytes"). Based on the results obtained during the current study we are using the TRI5 reporter strain to investigate the role of that gene in the barley/wheat-*F. graminearum* interaction and to learn more about the factors affecting and regulating production of DON under the conditions prevailing in the field and at processing of cereals, e.g. during malt production.

GLUCOSYLTRANSFERASES FROM *ARABIDOPSIS THALIANA* INACTIVATING THE *FUSARIUM* TOXINS DEOXYNIVALENOL AND ZEARALENONE

B. Poppenberger[1,3], F. Berhiller[2], D. Lucyshyn[1], R. Mitterbauer[1], W. Schweiger[1], R. Schuhmacher[2], R. Krska[2] and G. Adam[1*]

[1]BOKU – University of Natural Resources and Applied Life Sciences, Department of Applied Plant Sciences and Plant Biotechnology, Institute of Applied Genetics and Cell Biology, Vienna, Austria; [2]BOKU – University of Natural Resources and Applied Life Sciences, Department IFA-Tulln, Center for Analytical Chemistry, Tulln, Austria; and [3]Present Address: CNAP, Department of Biology, University of York, York YO10 5YW, UK
[*]Corresponding Author: PH: 0043-1-36006-6380; E-mail: gerhard.adam@boku.ac.at

ABSTRACT

During the infection of small grain cereals and maize *Fusarium graminearum* produces the mycotoxins deoxynivalenol (DON) and zearalenone (ZON). It has been demonstrated that the production of the trichothecene DON, which acts as an inhibitor of eukaryotic protein synthesis, contributes to the virulence of *Fusarium* (presumably by interfering with the expression of plant defense genes). ZON, which has very high estrogenic activity in animals, also seems to play a role in plant-pathogen interaction. We have searched for *Arabidopsis* genes which can inactivate these *Fusarium* toxins. A yeast strain highly sensitive to DON was used as a host for an *Arabidopsis thaliana* expression library and a UDP-glucosyltransferase (UGT) gene conferring resistance to DON (*DOGT1*) was identified (Poppenberger *et al.*, 2003). Overexpression of the *DOGT1* gene in *Arabidopsis* led to increased DON resistance of seedlings. The metabolite DON-3O-glucoside is inactive in inhibiting protein synthesis (tested with a wheat germ extract *in vitro* translation system). *DOGT1* is located in a cluster of 6 highly similar genes, but surprisingly the protein with the most closely related sequence is not protecting against DON. By making hybrid proteins and functional testing in yeast we characterized structural features essential for substrate specificity of these UGTs. Interestingly nivalenol, which has just one additional hydroxyl group, escapes detoxification.

We have also cloned an *Arabidopsis* UGT which converts ZON into ZON-4O-glucoside. While ZON shows strong binding to the estrogen receptor *in vitro*, this is not observed with ZON-glucoside. Expession of this UGT in an engineered yeast strain expressing the human estrogen receptor (hER), interferes with ZON-induced activation of hER-dependent reporter genes. This remarkable affinity can be exploited to produce ZON-glucoside in high yield by feeding ZON to the recombinant yeast.

We propose that the glucosides of DON and ZON produced by plants are a currently overlooked source of "masked mycotoxin". While the mycotoxin-glucosides escape standard analytical procedures, the toxic aglyca can be easily reactivated in the digestive tract.

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TRANSGENES IN WHEAT

S. Sato[1], X. Ye[1], B. Langston[2], J. Bohlmann[1], B. LaVallee[1], A. Mitra[1], S. Baenziger[1], M. Dickman[2] and T. Clemente[1*]

[1]Department of Agronomy and Horticulture; and [2]Department of Plant Pathology, University of Nebraska, Lincoln, NE, 68503-0915, USA
[*]Corresponding Author: PH: 402-472-1428; E-mail: tclemente1@unl.edu

ABSTRACT

Our wheat transformation team at the University of Nebraska-Lincoln (UNL) employs an *Agrobacterium*-mediated protocol to deliver transgenes to the crop. We recently completed a survey of 30 spring wheat genotypes for enhanced transformation frequencies. From this work a hard white genotype Xin chun 9 was identified that displayed improved transformability over Bobwhite. Current efforts are focused on evaluating a series of novel *Agrobacterium tumefaciens* strains in a comparative study with both Bobwhite and Xin chun 9.

We have been using this transformation system to evaluate potential antifungal transgenes in support of a collaborative effort targeting Fusarium Head Blight (FHB) resistance at UNL. To this end a total of 48 transgenic wheat lines harboring three novel negative regulators of programmed cell death, or a ribosomal inactivating protein have been handed-off to our wheat breeding program. More recently transgenic wheat lines carrying two additional negative regulators of programmed cell death and a synthetic antifungal lytic peptide have been produced. Field trials were conducted in the spring of 2004 on 28 transgenic lines carry three negative regulators of programmed cell death genes, *ced9*, *IAP* and *Bcl-xl*, along with lines harboring the ribosomal inactivating protein. Field plots were inoculated with *F. garminearum* just prior to anthesis. Data has been ascertained on agronomic parameters, days to anthesis, vigor and plant height, in addition to FHB severity and incidence.

OPTIMIZATION OF AN *AGROBACTERIUM*-MEDIATED TRANSFORMATION SYSTEM FOR DURUM WHEAT

Valluri V. Satyavathi[1], Prem P. Jauhar[2*] and Lynn S. Dahleen[2]

Dept. of Plant Sciences, North Dakota State University[1], USDA-ARS, Northern Crop Science Laboratory[2], Fargo, ND 58105, USA
[*]Corresponding Author: PH: (701) 239-1309; E-mail: prem.jauhar@ndsu.nodak.edu

OBJECTIVES

To optimize an *Agrobacterium*-mediated transformation system for durum wheat to facilitate incorporation of antifungal genes for resistance against Fusarium head blight.

INTRODUCTION

Durum wheat (*Triticum turgidum* L., $2n = 4x = 28$; AABB genomes) is an important cereal crop grown in the United States, Canada, and in some European countries. Several methods have been used for its genetic improvement. In recent years, genetic engineering has opened up new avenues for crop improvement and is a useful adjunct to conventional breeding. A prerequisite for application of such modern techniques is an efficient and reliable *in vitro* plant regeneration system. Gene transfers in plants have been achieved through direct DNA uptake, electroporation, microinjection, particle bombardment and *Agrobacterium*-mediated methods. We standardized an efficient regeneration system for commercial durum wheat cultivars (Bommineni and Jauhar, 1996; Satyavathi et al., 2004) and by using particle bombardment produced transgenic durum with marker genes (Bommineni et al., 1997) and antifungal genes (Satyavathi and Jauhar, 2003). Transgenic durum has now been produced in other laboratories (He et al., 1999; Pellegrineschi et al., 2002). In bread wheat, partial FHB resistance was achieved by expressing pathogenesis-related proteins using particle bombardment, but this technique was hampered by multiple copy gene insertions and gene silencing (Anand et al., 2003). Compared to direct gene transfer techniques, *Agrobacterium*-mediated transformation offers a number of advantages, including potentially low copy number and preferential integration into transcriptionally active regions of the chromosome (Hu et al., 2003). So far, an *Agrobacterium*-mediated transformation system has not been reported for durum wheat. Therefore, we attempted to optimize the conditions for *Agrobacterium* mediated transformation of the commercial durum wheat cv. Maier using marker genes.

MATERIALS AND METHODS

Plant material and preculture - An agronomically superior durum cultivar, Maier was used for transformation. Spikes were harvested 14 days post anthesis and the spikelets were surface sterilized and cultured as described by Bommineni and Jauhar (1996). The callus induction medium was supplemented with 2.0 mg L^{-1} dicamba. The cultures were incubated in the dark at 25 ± 2°C for 1-14 days depending on the experiment performed.

Agrobacterium tumefaciens strain, plasmid, and culture - A disarmed *Agrobacterium tumefaciens* strain AGL1 harboring pDM805 was provided by CSIRO Plant Industry, Canberra, Australia (Fig. 1). The binary vector pDM805 contains the phosphinothricin acetyltransferase (*bar*) gene under the control of the promoter from the maize ubiquitin 1 (*Ubi1*) gene and a terminator sequence from the *A. tumefaciens* nopaline synthase (*nos*) gene; the β-glucuronidase gene *uidA* (*gus*) under the control of the promoter from the rice actin 1 (*Act1*) gene and a terminator sequence from the rice ribulose bis-phosphate carboxylase/oxygenase (*rbcS*) gene. A full strength *Agrobacterium* suspension was obtained a day before transformation as described by Tingay et al. (1997).

Acetosyringone treatment and particle bombardment - Acetosyringone effects on trans-formation ef-

ficiency were studied by comparing *Agrobacterium* suspension without the chemical to suspension treated with 200 µM acetosyringone prior to infection. The explants were then infected for about 1 h. To study the effect of particle bombardment on the extent of infection, the scutella were precultured for 14 days and wounded by bombardment with gold particles. About 30-50 explants were bombarded with 0.3 mg of gold particles (1.0 µM) using a BioRad PDS-1000 biolistic device with 1,100 psi rupture disc and compared to unwounded explants.

Inoculation and co-cultivation - Isolated scutella precultured for 1 day and 14 days were used for transformation. The explants were immersed in full strength *Agrobacterium* suspension in a Petri dish for half an hour (1 h when acetosyringone was used in the suspension) and then transferred without rinsing, with scutellar surface placed in contact with the callus induction medium. Co-cultivation was carried out at 25 ± 2°C in darkness for about 2-3 days.

Selection and plant regeneration - After co-cultivation, the explants were washed thrice with sterile distilled water in a Petri dish and blotted on sterile Whatman filter paper. The explants were then plated on selection medium, which was same as callus induction medium but was supplemented with 150 mg L^{-1} Timentin and 5.0 mg L^{-1} bialaphos. Explants were maintained on selection medium for 3-4 weeks at 25 ± 2°C in darkness, after which they were transferred to regeneration medium (selection medium without growth regulators). The culture conditions and regeneration procedure were same as described by Satyavathi et al. (2004).

Histochemical GUS assay - T-DNA delivery into explant tissues was determined after 1-3 weeks of culture on selection medium using the histochemical GUS assay according to Bommineni et al. (1997). Explants with blue spots and the number of blue spots per explant were counted under a stereomicroscope.

Statistical analyses - For studying the effect of acetosyringone and bombardment on DNA delivery, we compared GUS expression among the explants. Each treatment had three replications and at least 50 explants (4 from each Petri dish) per treatment were sacrificed for GUS assays. As the data on the number of explants with GUS spots and the number of spots per explant for each treatment were not normally distributed, analysis of variance was done using PROC CATMOD (SAS version 8.2, 2001).

RESULTS

Preliminary tests were performed to compare the responses of the scutella that were precultured for 1 day vs 10-14 days. After 1-2 days of co-cultivation with *Agrobacterium* and on transfer to selection medium, only 10% of the scutella precultured for 1-day initiated calli. In the case of 10-14 day precultured scutella, about 70% of them developed callus over the cut surface within a week and later developed callus around the periphery of the scutellum. After 3 weeks on selection medium, 511 of 725 scutella co-cultivated were resistant to bialaphos selection. GUS assays done 7 days after co-cultivation showed GUS spots all over the scutellum surface but most of the spots were localized on the areas starting to form callus, usually at the periphery of the scutellum (Fig. 2A & B). In subsequent subcultures, the proliferating callus was selected and brown callus was discarded. After 4 weeks on selection medium, the embryogenic callus was transferred to regeneration medium supplemented with 5.0 mg L^{-1} bialaphos. Out of 725 scutella infected, only 3 plantlets were regenerated at the end of 3-4 weeks with a transformation frequency of 0.4%. Various treatments like increasing co-culture duration, adding acetosyringone in the *Agrobacterium* suspension, and bombarding the explants with gold particles before infection, had differential effects on transformation efficiency as follows:

Duration of co-culture - About 82% of the scutella that were co-cultivated for 2 days were resistant to bialaphos selection. When the co-cultivation was extended to 3 days, an overgrowth of the bacteria was observed on 50% of the scutella and the percentage of resistant scutella decreased to 56%.

Effect of acetosyringone - GUS expression was detected in all the tissues 3 wk after co-cultivation either in the presence or absence of the pretreatment.

The scutella that were treated with acetosyringone did not differ for GUS expression in terms of the number of explants with GUS spots and the number of GUS spots per explant compared to those infected with *Agrobacterium* suspension without acetosyringone (Table 1).

Effect of bombardment - The explants that were injured by bombarding with gold particles showed significantly greater number of explants with GUS spots ($p < 0.05$) and also significantly greater GUS spots per explant ($p < 0.01$) than those that were not wounded prior to infection (Table 1).

DISCUSSION

This work presents the first report on *Agrobacterium*-mediated transformation of durum wheat. A prerequisite for development of *Agrobacterium*-mediated transformation is the establishment of optimal conditions for T-DNA delivery into tissue from which whole plants can be regenerated. Based on our previous findings, we selected durum cultivar Maier for transformation with *Agrobacterium*. In general, model genotypes amenable to tissue culture or to microprojectile transformation have worked well for *Agrobacterium*-mediated transformation in several crops like wheat, maize, barley, and sugar cane (Cheng et al., 2004). The isolated scutella are known to be choice explants for many cereals including durum wheat and have been successfully used for regeneration and transformation experiments. In the present study, we found that 10-14 days preculture of explants increases transformation efficiency. Similar results were observed in wheat where longer precultures resulted in efficient T-DNA delivery (Cheng et al., 1997; Wu et al., 2003). We obtained a transformation frequency of 0.4% which is comparable to that reported in other cereals. In wheat, transformation frequencies ranged from 0.3-4.3% and were increased to 10.5% by desiccation of precultured embryos (Cheng et al., 2004).

Chemicals such as acetosyringone for *vir* gene induction are recommended in most of the monocot transformation protocols. We used a 200 µM acetosyringone treatment prior to infection. The presence of acetosyringone did not increase GUS expression. The addition of acetosyringone at a concentration of 150 to 200 µM during preculture or co-culture increased the number of transformed cells in rice (Hiei et al., 1994), barley (Tingay et al., 1997), and wheat (Cheng et al., 1997). In the present study, wounding precultured scutella with gold particles before infecting the explants with *Agrobacterium*, increased the GUS expression significantly. Similar results were observed in barley (Tingay et al., 1997).

The advantage of *Agrobacterium*-mediated transformation over particle bombardment is that this method is simple and cost effective. We optimized conditions for *Agrobacterium*-mediated transformation using scutella of the cultivar Maier and also studied the effects of various pretreatments that could enhance infection and T-DNA delivery. Further experiments are needed to increase regeneration from transformed callus.

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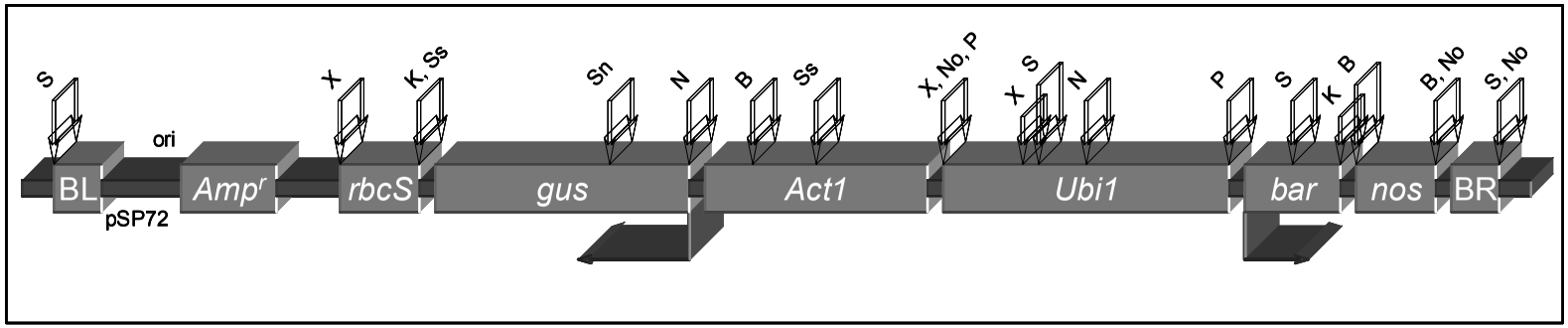

Figure 1. Structure and restriction map of the cereal transformation vector pDM805 (Tingay et al., 1997).

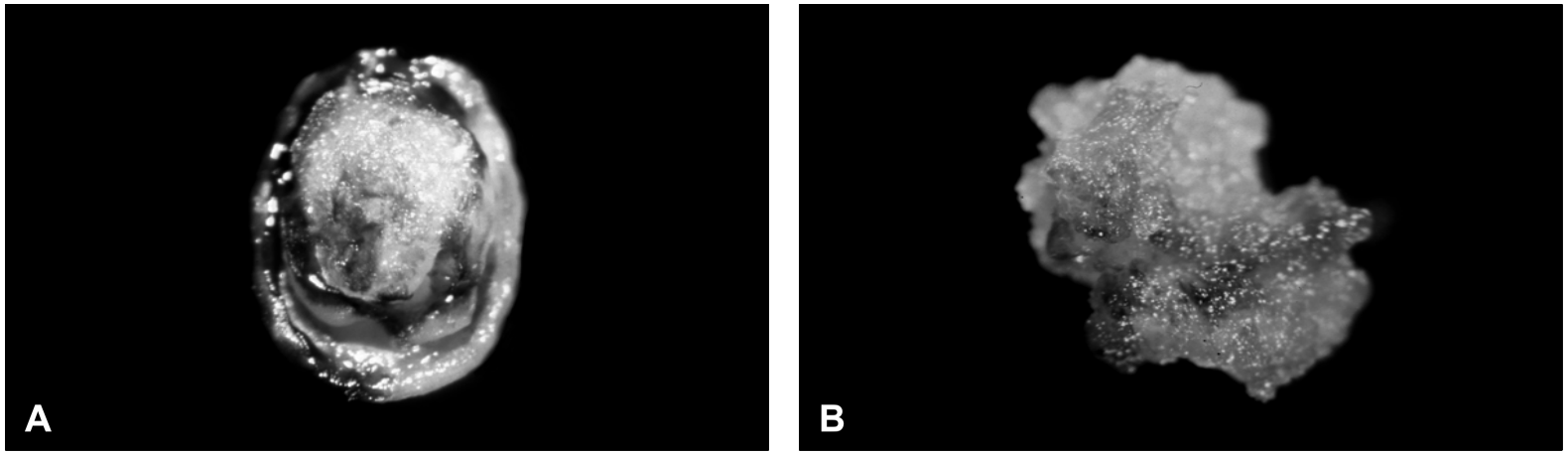

Figure 2. GUS expression in the scutellum one week after co-cultivation with *Agrobacterium.* GUS expression in callus that was resistant to 5.0 mg L-1 bialaphos three weeks after co-cultivation with *Agrobacterium*.

Table 1. Effect of different pretreatments on GUS expression in 14-day precultured durum wheat scutella infected with *Agrobacterium tumefaciens* strain AGL1 harboring pDM805.

Treatment	Number of explants	Proportion of explants with GUS spots (%)	GUS spots /explant
Acetosyringone			
Untreated	52	26/52 (50.00)	3.35
Treated	52	34/52 (65.38)	4.20
Bombardment			
Untreated	72	17/72 (23.61)	1.47
Bombarded	72	31/72 (43.05)*	2.54 **

* Chi-square value of 0.0194 was significant at p = 0.05 level
** Chi-square value of 0.0037 was significant at p = 0.01 level

DEVELOPMENT OF TISSUE-SPECIFIC PROMOTERS FOR TARGETING ANTI-FUSARIUM GENE EXPRESSION

R.W. Skadsen[1*], M.L. Federico[2], T. Abebe[3] and M. Patel[4]

[1]U.S. Dept. of Agriculture, ARS, Cereal Crops Res. Unit, 501 Walnut St., Madison, WI 53711, USA; [2]University of Wisconsin, Agronomy Dept., 1575 Linden Dr., Madison, WI 53706-1590, USA; [3]University of Northern Iowa, Dept. of Biology, 144 McCollum Science Hall, Cedar Falls, IA, USA; and [4]University of Wisconsin, Plant Pathology Dept., 1630 Linden Dr., Madison, WI 53706-1589, USA
[*]Corresponding Author: PH: (608)262-3672; E-mail: rskadsen@wisc.edu

ABSTRACT

We identified lemma and pericarp epithelium tissues as rapidly infected by *Fusarium graminearum*. Genes specifically expressed in these tissues were identified and cloned so that promoters of selected genes could be used to express antifungal protein genes. These included a lipid transfer protein homologue (*Ltp6*), highly expressed in the pericarp epithelium but not in vegetative leaves, and a jacaline-like gene, *Lem2*, preferentially expressed in the lemma/palea, compared with the flag leaf. *Ltp6* is also expressed in coleoptiles and embryos; mRNA levels increase in response to salt, cold, abscisic acid and salicylic acid in a pattern distinct from other barley *Ltp*s. Transient expression analysis of the promoter showed that 192 bp of upstream sequence confers tissue-specific expression and retains most promoter activity. Stable barley transformants have been produced with a 247 bp promoter fused to a *gfp* reporter gene. In these, *gfp* expression is strong in the epicarp, embryo and coleoptile, but it is not found in other tissues. *Gfp* expression was detected during spike development, from early ovary differentiation through its final expression in the epicarp and during embyogenesis and germination in the coleoptile, reproducing the expression pattern of the native gene. *Lem2* is specifically expressed in the lemma/palea and coleoptile. SA induces *Lem2* within 4 h, suggesting that it is a defensive gene. Promoter deletion studies showed that the tissue-specificity and promoter activity are conferred by a short 5' proximal region from -75 to +70. Stable transformants were produced with the "full-length" 1414 bp promoter and 5' promoter deletions fused to *gfp*. G*fp* expression occurred in the lemma/palea and coleoptile, but it also unexpectedly occurred in the epicarp and ligules. Lack of methylation in the epicarp may account for expression in the epicarp.

TARGETING OF ANTI-FUSARIUM GENE EXPRESSION IN BARLEY

R.W. Skadsen[1*], M.L. Federico[2], T. Abebe[3] and M. Patel[4]

[1]U.S. Dept. of Agriculture, ARS, Cereal Crops Res. Unit, 501 Walnut St., Madison, WI, 53711, USA; [2]Univ. of Wisconsin, Agronomy Department, 1575 Linden Dr., Madison, WI, 53706-1590, USA; [3]Univ. of Northern Iowa, Department of Biology, 144 McCollum Science hall, Cedar Falls, IA, USA; and [4]Univ. of Wisconsin, Plant Pathology Department, 1630 Linden Dr., Madison, WI 53706-1589, USA

[*]Corresponding Author: PH: (608)262-3672; E-mail: rskadsen@wisc.edu

ABSTRACT

We identified lemma and pericarp epithelium (epicarp) tissues as rapidly infected by a strain of *Fusarium graminearum* transformed with the green fluorescent protein gene (*gfp*). The fungus colonized the lemma in 48 h, but it colonized the brush hairs at the seed tip within 7 h and rapidly grew downward along the epicarp and more slowly inward through the cross cells (Skadsen and Hohn, PMPP 64:45-53, 2004). Genes specifically expressed in the lemma (Abebe et al., Crop Sci. 44:942-950, 2004) and epicarp were identified and cloned so that promoters of selected genes could be used to express antifungal protein genes in these susceptible tissues. Tissue-specific genes included a lipid transfer protein homologue (*Ltp6*), highly expressed in the pericarp epithelium but not in vegetative leaves, and a jacaline-like gene, *Lem2*, preferentially expressed in the lemma/palea, compared with the flag leaf. *Ltp6* is also expressed in coleoptiles and embryos; mRNA levels increase in response to salt, cold, abscisic acid and salicylic acid (SA) in a pattern distinct from other barley *Ltp*s. Transient expression analysis of the promoter showed that 192 bp of upstream sequence confers tissue-specific expression and retains most promoter activity. Stable barley transformants have been produced with a 247 bp promoter fused to a *gfp* reporter gene (Federico et al., PMB, in press). In these, *gfp* expression is strong in the epicarp, embryo and coleoptile, but it is not found in other tissues. *Gfp* expression was detected during spike development, from early ovary differentiation through its final expression in the epicarp, and during embyogenesis and germination in the coleoptile, reproducing the expression pattern of the native gene. *Lem2* is specifically expressed in the lemma/palea and coleoptile. SA induces *Lem2* within 4 h, suggesting that it is a defensive gene. Deletion studies showed that tissue-specificity is conferred by a short 5' proximal region from -75 to +70 (Abebe *et al*., Planta, in press) . Stable transformants were produced with the "full-length" 1414 bp promoter and 5' promoter deletions fused to *gfp*. G*fp* expression occurred in the lemma/palea and coleoptile, but it also unexpectedly occurred in the epicarp, perhaps due to a lack of methylation. In the lemmas, a developmental transition occurred wherein *gfp* was first expressed in the mesophyll cells; this was gradually replaced by expression in specialized cork cells of the epidermis. An additional promoter, *Lem1*, was produced by Sathish Puthigae and showed lemma/palea-specific expression in transient assays (Skadsen et al., PMB, 49:545-555, 2002).

A NOVEL STRATEGY FOR TRANSGENIC CONTROL OF FUSARIUM HEAD BLIGHT IN WHEAT

M. Somleva* and A. Blechl

USDA-ARS, Western Regional Research Center, Albany, CA 94710, USA
*Corresponding Author: PH (510) 559-5673; E-mail: msomleva@pw.usda.gov

ABSTRACT

Changes in agricultural practices (e.g., minimal tilling) during the past two decades combined with changing climate conditions have dramatically altered crop susceptibility to Fusarium head blight (FHB) or scab. FHB may lead to direct yield losses of 5-20% worldwide in average epidemic years, but losses as high as 60-70% have also been reported. Host plant resistance, the most cost-effective way to fight the disease, in available wheat germplasm is only partial and has been difficult to incorporate into cultivars adapted for regional growth in the U.S. Our goal is to achieve FHB resistance by employing plant genetic transformation, a potentially powerful tool for transgenic control of fungal diseases in cereals. We selected three candidate anti-*Fusarium* (AF) genes: *Aspergillus* glucose oxidase (*GO*) and barley peroxidases (*Prx7* and *Prx8*) based on their association with an array of naturally occurring plant defense mechanisms. Glucose oxidase is an apoplastic enzyme that catalyzes oxidation of β-D-glucose, generating H_2O_2, a compound with multiple functions in plant defense. Induction of the peroxidases Prx7 and Prx8 has been correlated with the appearance of antifungal compounds and papillae structures, respectively, in barley leaves exposed to powdery mildew. We inserted the coding regions of these genes into our vector that contains the barley *Lem1* promoter, which we have previously shown is active in the outer organs of transgenic wheat florets from anthesis to the soft dough stage of kernel development. This activity pattern makes it an excellent candidate for targeting AF gene expression to the path of *Fusarium* invasion. We have generated 100 transgenic wheat lines carrying the Lem1::PRX and/or Lem1::GO constructs. The *in situ* methods used for expression analyses of the primary transformants revealed that the transgene-encoded proteins are accumulated either in the extracellular space (GO and Prx8) or in the cells (Prx7) of the spike tissues and were not present in developing grain. The possible synergistic effect of these enzymes on improving host resistance to initial fungal infection and pathogen spread will be discussed. If our strategy is successful, the lack of recombinant proteins in the grain will minimize concerns about the safety of foods derived from these wheats and facilitate their approval by regulators and acceptance by consumers.

CHARACTERIZATION OF ORGAN SPECIFIC PROMOTERS IN TRANSGENIC WHEAT

M. Somleva* and A. Blechl

USDA-ARS, Western Regional Research Center, Albany, CA 94710, USA
*Corresponding Author: PH (510) 559-5673; E-mail: msomleva@pw.usda.gov

OBJECTIVE

To identify promoters suitable for targeting anti-*Fusarium* gene expression to wheat tissues surrounding the developing seed.

INTRODUCTION

Genetic engineering is the most promising approach to increase plant resistance to fungal pathogens, including *Fusarium*. The effectiveness of an antifungal gene *in planta* is determined by its expression levels in the crucial host tissues and by the timing of its expression such that suitable levels of the encoded protein accumulate before the infection (Dahleen et al., 2001). At present, constitutive promoters are widely used to achieve high expression levels throughout most tissues of the plant. If only specific tissues need to be protected or antifungal compounds need to be expressed at certain targeted sites, the use of specific promoters is recommended (Punja, 2001). Expression of anti-*Fusarium* (AF) genes in the glume and lemma is desirable for both wheat and barley, because these organs comprise the outer most protective barrier encasing the reproductive organs. In this study we present the organ- and developmental specificity of the promoter of a maize glutamine synthase gene, *GS*, and the promoter of a barley floret-expressed gene, *Lem1*, in stable hexaploid wheat transformants.

MATERIALS AND METHODS

Vector and plasmid constructs: The following plasmids were used for wheat transformation: pGS176 and pGS177 carrying a 664-bp fragment of the promoter of the maize $GS_{1\text{-}2}$ gene fused to the *uidA* coding region (GUS) and the first introns of the native gene and of the maize alcohol dehydrogenase (*ADH)* gene, respectively (Muhitch et al., 2002); and pBSD5sGFP carrying a 1400-bp fragment containing the *Lem1* promoter and a partial N-terminal coding region fused to the coding sequence for the green fluorescent protein (Lem1::GFP, Skadsen et al., 2002). For comparative studies of the promoter activity patterns, the plasmid pAHC15 carrying the *uidA* gene driven by the maize *Ubi1* promoter and first intron (UBI::GUS) was also used (Christensen and Quail, 1996).

Generation of primary transformants and monitoring of the reporter gene expression: Transient expression assays and wheat transformation were performed by particle bombardment of immature embryos of cv. Bobwhite. Stably transformed plants were identified as described (Okubara et al., 2002). GFP fluorescence in various tissues of transgenic plants and *in vitro* cultures was monitored using an Olympus SZX stereomicroscope equipped with an SZX-RFL fluorescence attachment and a DP11 digital camera. GUS activity was detected according to Hänsch et al. (1995).

Construction of cloning vectors: To make pBGS9Lem1, a 1067-bp fragment containing the *Lem1* promoter was PCR-amplified from the plasmid pBSD5sGFP (Skadsen et al., 2002) using Pfu Polymerase (Stratagene) and primers 5'-GATAAGCTTGGGATGTC-3' and 5'-ACGGATATCTGCGGTTGAAG-3' with 5' extensions to add *Hind*III and *Eco*RV restriction sites, respectively. After complete digestion with *Hind*III, the resulting fragment was ligated with a 3935-bp restriction fragment containing pBGS9 (Spratt et al., 1986) and the NOS 3' transcriptional terminator sequence. The latter piece of DNA had been prepared from the monocot transformation vector pUBK (Okubara et al., 2002). To make pBGS9Lem1ADHi1, the first in-

tron of the maize alcohol dehydrogenase gene, *ADH*, was PCR-amplified using the plasmid pGS177 (Muhitch et al., 2002) as a template. The resulting 601-bp fragment was inserted into the *Eco*RV site of pBGS9Lem1. A unique *Sma*I restriction site separates the *ADH* intron and the NOS 3' region.

RESULTS AND DISCUSSION

In wheat, the period of susceptibility to head infection by *Fusarium* lasts from anthesis (the time point when the anthers extrude from the spikes) through the dough stage of kernel development. To identify a promoter suitable for expression of anti-*Fusarium* genes, the activities of reporter genes GUS and GFP fused to the maize *GS* and barley *Lem1* promoters, respectively, were monitored during growth and development of primary wheat transformants and their progeny. The *GS* promoter is only expressed in the pericarp and in the scutellum of mature embryos (Fig. 1). Thus, it is not suitable for use in anti-*Fusarium* constructs. In transgenic plants carrying both Lem1::GFP and UBI::GUS, we compared the activity patterns of the two promoters by monitoring the expression of both reporter genes during plant development (Fig. 2). We observed no GFP fluorescence in vegetative organs, indicating that the *Lem1* promoter was not active in these tissues. This is in accordance with the data for its organ- and developmental specificity in barley (Skadsen et al., 2002). In floret tissues, we detected no GFP fluorescence before anthesis, demonstrating that the *Lem1* promoter did not function before this stage (data not shown). In contrast, strong UBI-driven GUS activity was detected in the young ovary and anthers (data not shown). At anthesis, UBI is active in the reproductive organs (Fig. 2A), while the *Lem1* promoter drove high levels of *gfp* expression only in the organs surrounding the developing floret (Fig. 2A). (The autofluorescence of the anthers was also seen in control plants.) These findings indicate that the *Lem1* promoter is active during the same period in spike development in transgenic wheat as it is in its native context in barley. No GFP fluorescence was seen in developing seeds (Fig. 2B and C). In contrast, GUS activity driven by the UBI promoter was detected in the seed coat during the earliest stages of grain development – watery ripe and soft dough stages (Fig. 2B and C, respectively).

The relative strength of the barley *Lem1* promoter was assessed in a transient assay (Fig. 3). Transient *gfp* expression was first observed in wheat embryos 10 h after bombardment (Fig. 3B), while *uidA* expression driven by maize UBI was detected within 2 h (Fig. 3A). This finding indicates that that *Lem1* is less active than UBI, which is one of the strongest of cereal promoters characterized to date. Approximately the same difference in *gfp* transient expression under the control of *Lem1* and UBI promoters was shown by Skadsen et al. (2002) in bombarded spikes of wheat and barley.

Results from these comparative studies suggest that, due to its organ specificity and moderate strength, the barley *Lem1* promoter would be an excellent choice to target anti-*Fusarium* gene expression to wheat tissues surrounding the developing seed at anthesis, while excluding transgene-encoded foreign proteins from the edible grain. To facilitate its use for this and other purposes, we constructed the cloning vectors pBGS9Lem1 carrying the *Lem1* promoter and the NOS 3' terminator sequence (Fig. 4A) and pBGS9Lem1ADHi1, in which the first intron of the maize *ADH* gene was fused to the *Lem1* promoter (Fig. 4B). Both vectors have unique blunt-end restriction sites that can be used for insertion of any coding sequence. Recently, we have successfully employed pBGS9Lem1 to express candidate anti-*Fusarium* genes in transgenic wheat.

ACKNOWLEGEMENTS

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Figure 1. Activity of the *GS* promoter in developing florets and seeds in transgenic wheat. (*GUS activity visible in color photographs is indicated by* arrows.) **A** No GUS activity was observed in a young ovary and anthers. **B** GUS activity in a maturing ovary in a spikelet after pollination. There was no staining in the outer floral organs. **C** GUS activity in the pericarp. **D** and **E** No *uidA* expression was detected in immature embryos. **F** GUS activity in the scutellum of a mature embryo.

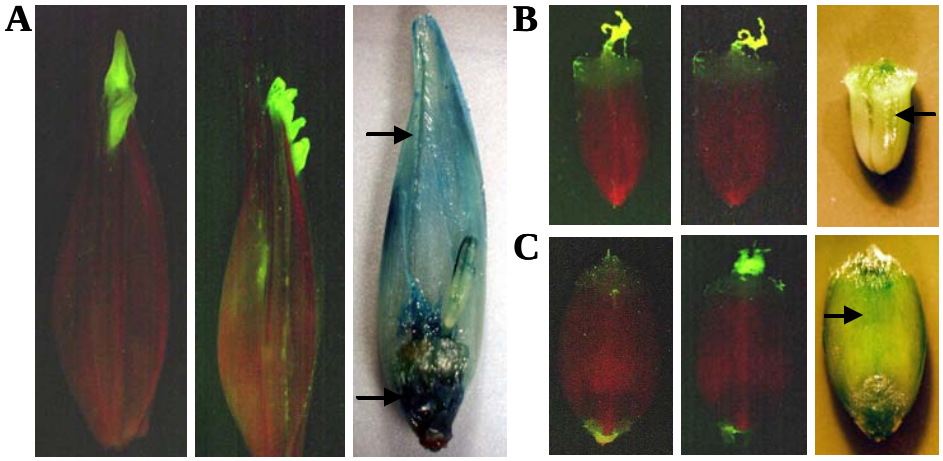

Figure 2. Activity patterns of the barley *Lem1* and maize UBI promoters during development of wheat florets and grain. Reporter gene expression was monitored in stable wheat transformants carrying Lem1::GFP and UBI::GUS, either by direct fluorescence (GFP) or by histochemical staining (GUS). Each panel shows developing florets and seeds from non-transformed plants under UV light (*left*) and GFP fluorescence (*center*) and GUS activity (*right*) in the same type of specimens from transgenic plants. (*GFP fluorescence is visible as very light areas. GUS activity visible in color photographs is indicated by* arrows.) **A** Florets at anthesis. Note the lack of GFP fluorescence in the outer floret organs of the control. **B** Developing kernels at a watery ripe stage. **C** Wheat grain at a soft dough stage. Note that the mature anthers and hairs of the caryopsis brush show strong autofluorescence under these conditions.

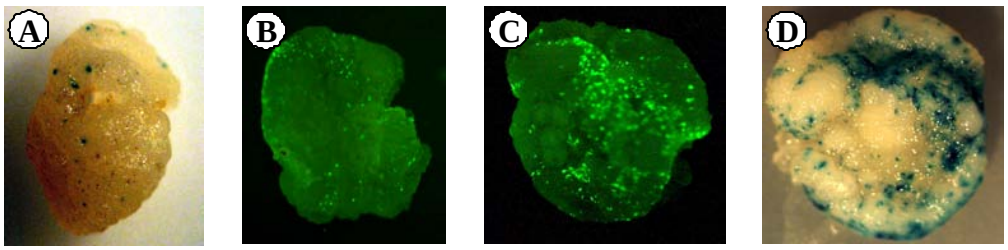

Figure 3. Activity of *Lem1* and UBI promoters during callus initiation and culture. Isolated zygotic wheat embryos 21 DAA were co-bombarded with Lem1::GFP and UBI::GUS. **A** GUS activity in an embryo 2 h after bombardment (*dark spots)*. **B** Expression of *gfp*10 h after bombardment *(lighter spots).* **C** GFP fluorescence in a callus after one week of culture on the recovery medium. **D** The callus shown in Fig. 3C after staining for GUS activity.

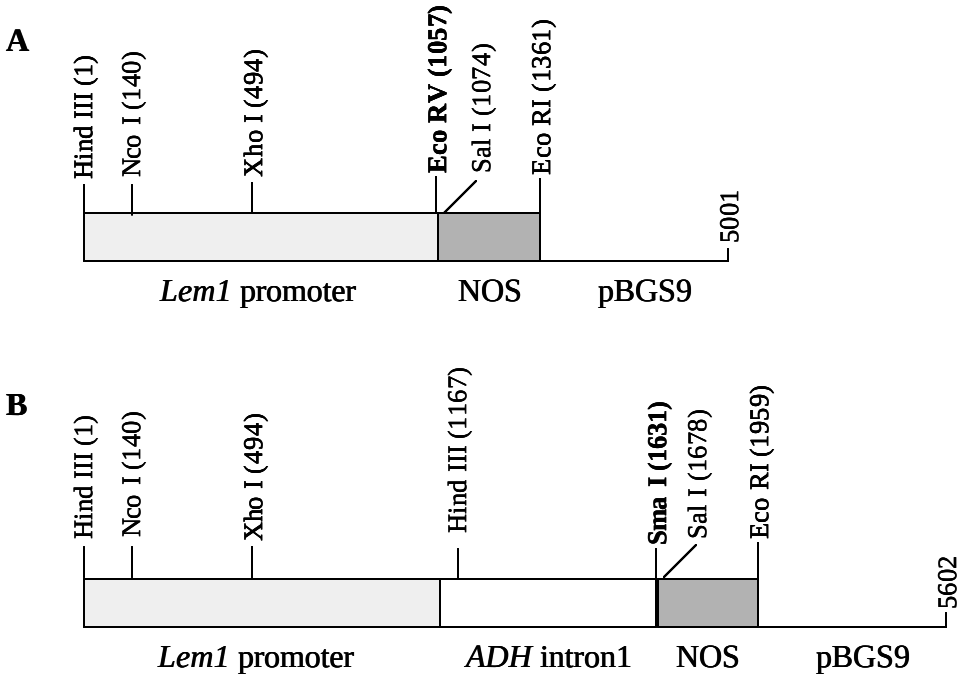

Figure 4. Linear diagrams of the cloning vectors. **A** The plasmid pBGS9Lem1 features the barley *Lem1* promoter and nopaline synthase terminator (NOS). **B** The plasmid pBGS6Lem1ADH1 contains the *ADH* first intron inserted between the promoter and NOS. The unique *Eco*RV (**A**) and *Sma*I (**B**) sites were created to facilitate insertion of any coding region as a blunt-ended fragment. The locations of some other restriction sites are shown. The pBGS9 portion is not to scale.

COMBINED EXPRESSION OF CANDIDATE ANTI-*FUSARIUM* GENES IN WHEAT SPIKELETS

M. Somleva* and A. Blechl

USDA-ARS, Western Regional Research Center, Albany, CA 94710, USA
*Corresponding Author: PH (510) 559-5673; E-mail: msomleva@pw.usda.gov

ABSTRACT

Fusarium head blight (FHB) or scab is one of the most destructive diseases of wheat, causing significant reductions in grain yield and quality. Although partial resistance has been identified in wheat varieties, no sources of immunity to *Fusarium* have yet been found. Genetic engineering is a promising method to create new sources of wheat germplasm with host plant resistance to scab. Expression of genes conferring resistance to FHB in wheat is desired in the glume and lemma, because these organs comprise the outer most protective barrier encasing the reproductive organs. Our objectives were to 1) introduce genes encoding recombinant antifungal proteins into bread wheat, *cv*. Bobwhite and 2) characterize their expression in *in vitro* cultures and stable transformants. A cloning vector carrying the barley *Lem1* gene promoter was constructed. We have previously shown that the *Lem1* promoter is active in wheat florets from anthesis to the soft dough stage of kernel development, making it an excellent candidate for targeting antifungal gene expression to the path of *Fusarium* invasion. Coding regions of genes selected for their ability to induce an array of naturally occurring plant defense mechanisms – the *Aspergillus* glucose oxidase gene and two barley peroxidase genes, *Prx7* and *Prx8* - were fused to *Lem1* and introduced into wheat immature embryos by particle bombardment. Functional analyses of the expression cassettes were performed by transient assays. The activities of the transgene-encoded proteins were studied in spike tissues of primary transformants and their progeny using enzyme assays. The employed *in situ* methods revealed that the recombinant peroxidases were present in the organs surrounding the developing floret at anthesis, but not in the developing grain.

EXPRESSION PATTERNS OF CHITINASE AND THAUMATIN-LIKE PROTEINS IN THREE TRANSFORMATION EVENTS OF BARLEY (*HORDEUM VULGARE* CV. CONLON)

D.J. Tobias[1*], C. Pritsch[1], L.S. Dahleen[2] and A.K. Jha[1]

[1]Department of Plant Sciences, North Dakota State University; and [2]Cereal Crops Research Unit,USDA-ARS NCSL, Fargo, ND 58105, USA
*Corresponding Author: PH: (701)239-1345; E-mail: dennis.tobias@ndsu.nodak.edu

OBJECTIVE

To analyze the protein expression patterns in barley transformed with two antifungal genes.

INTRODUCTION

Fusarium head blight (FHB), predominantly caused by *Fusarium graminearum*, is a devastating disease in cereal grains including barley. Resistant sources available to plant breeders are multigenic and provide only partial protection from FHB. Resistance to FHB might be achieved by overexpressing pathogenesis-related (PR) proteins that degrade structural components of the fungal pathogen. Currently, there are no reports of barley genotypes that are highly resistant to FHB. Combinations of antifungal and antitoxin genes are likely to offer a greater degree of resistance than transgenic plants containing single genes. Delayed FHB development has been reported in transformed spring wheat overexpressing *tlp* (Chen et al. 1999), and in plants co-expressing chitinase and glucanase genes (Zhu et al. 1994, Jongedijk et al. 1995, Anand et al. 2003), demonstrating the potential role of antifungal proteins against FHB. The *tlp* is a membrane permeabilizing protein while *chi* catalyzes the degradation of chitin, a cell wall component of most filamentous fungi. Previously, the barley malting cultivar Conlon was transformed with two antifungal genes, *chi* and *tlp* by particle bombardment (Dahleen and Manoharan 2003) and 58 plants from three transformation events were obtained and analyzed for transgene integration and expression. In the the present report, T_3 progenies from the three transformation events were further analyzed for chitinase (*chi*) and thaumatin-like protein (*tlp*) expression in both leaf and spike tissues.

MATERIALS AND METHODS

Transformation and regeneration - Immature embryos from the malting cultivar Conlon (two-rowed barley) were cultured on callus induction medium (Dahleen and Bregitzer 2001) and transformed with the antifungal genes, *chi* and *tlp,* by particle bombardment (Lemaux et al. 1996). Plasmids used for co-bombardment, pAHRC-*tlp* harboring the *tlp* gene and pAHG11 containing the *chi* gene, were provided by Dr. S. Muthukrishnan (Kansas State Univ.). Both plasmids contain the *bar* gene for bialaphos selection. The *tlp* and *chi* genes are under the control of the maize *ubil* promoter and terminated by *nos*. Transgenic plants were regenerated as described by Manoharan and Dahleen (2002).

T_3 progeny analyses - DNA from leaf and spike tissues was prepared using a modified CTAB method. PCR was carried out to determine the presence of *chi* and *tlp* genes in homozygous transgenic materials. PCR products were separated by agarose gel (1%) electrophoresis. Southern hybridization was carried out to confirm the *chi* or *tlp* transgene integration pattern in the three events. Genomic DNA was digested with *Hin*dIII, electrophoresed on a 1% agarose gel and transferred onto Hybond N+ membrane. Probes used were full length inserts (both 1.1 kb) released from the plasmids by *Bam*HI/*Hin*dIII (*chi*) or *Pst*I (*tlp*) digests and labeled with ^{32}P dCTP using the Redi-Prime Labeling Kit (Amersham Pharmacia, Buckinghamshire, England). Total soluble protein was prepared from young leaf and spike tissues of transgenic and control plants. Western analyses were conducted using the Immun-Blot Colorimetric Assay (Bio-Rad, Hercules, CA). Antibodies for *tlp* and *chi* were provided by Dr. R. Skadsen (USDA-ARS, Madison, WI) and Dr. S.

Muthukrishnan (Kansas State Univ.), respectively. Reverse-transcriptase (RT)-PCR was conducted using the One Step RT-PCR System with Platinum Taq DNA polymerase (Invitrogen).

RESULTS AND DISCUSSION

PCR analysis for *tlp* on the T_3 progenies of three transformation events (Events 1, 2 and 3) was in agreement with the previous report of Dahleen and Manoharan (2003). All transgenic events showed the presence of the *tlp*. While *chi* was detected in Event 2, PCR detection of *chi* in Events 1 and 3 was not consistent (data not shown). Southern hybridization was carried out to confirm the transgene integration pattern in the three events. All transgenic lines tested from each event were found positive for *tlp* but only Event 2 showed stable integration of the *chi* transgene (Fig. 1).

Previous Western analysis of T_2 plants from three transformation events (Dahleen and Manoharan 2003) suggested that both transgenes were silenced in two events (Events 1 and 3). Our present results confirmed the presence of a 26 kD rice *chi* in Event 2 leaves and spikes that was not detected in transgenic lines from the other two events (Fig. 2a). Aside from a 35-kD putative barley *chi* found in leaves from all transgenic plants and the wildtype, other chitinase bands of 25 and 31 kD were detected. Although the 35-kD *chi* band found in leaves was also detected in Event 2 spikes, a smaller band (ca. 34 kD) was present in Events 1 and 3 spikes (Fig. 2b). In all three events including the non-transgenic Conlon spikes, at least one band of *chi* close to 25 kD was present.

The expected 23-kD protein of the rice *tlp* was highly expressed in Event 2 leaves but had lower levels in spikes of the other two events (Fig. 2c). In spikes (Fig. 2d), a native *tlp* of approximately 23 kD is expressed in wildtype Conlon and transgenics, which comigrates with the rice *tlp*, making transgene expression levels difficult to determine. A putative 24-kD protein native to barley as well as smaller proteins ranging from 13 to 18 kD were detected in leaves and spikes of all transgenic events and the wildtype.

It is possible that the bands detected in leaves or spikes other than the 26 kD rice *chi* or 23 kD rice *tlp* could be either isoforms that are native to barley or could have been derived by proteolytic processing. To further analyze the differential protein expression and isoforms in leaf and spike tissues of transgenic and wildtype Conlon, isoelectric focusing gel electrophoresis is currently underway. Northern blot analyses are being used to confirm the mRNA expression level of *chi* and *tlp* in all transformation events using gene specific probes. Initial RT-PCR showed *chi* transcripts in leaves for all events (data not shown) which could be due to the presence of another chitinase endogenous to barley since the primer pairs amplify a short (372 bp) region of the gene that contains 240 bases having 84% sequence similarity with a barley (cv. NK1558) chitinase gene. RT-PCR analyses are being conducted using pertinent primer pairs to discriminate rice *chi* and *tlp* transcripts from corresponding transcripts that are endogenous to barley. Work is underway to optimize conditions for isoelectric focusing and RT-PCR.

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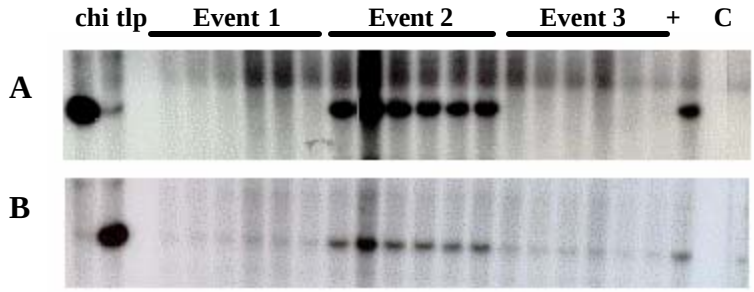

Figure 1. Southern analysis confirming the integration of (a) *chi* gene only in Event 2 (b) *tlp* in all three transformation events. Thirty µg of genomic DNA was loaded per la chi = wildtype Conlon DNA plus 250 pg chi (1.1 kb); tlp = wildtype Conlon DNA plus pg tlp (1.1 kb); + = positive control DNA; C = wildtype Conlon DNA.

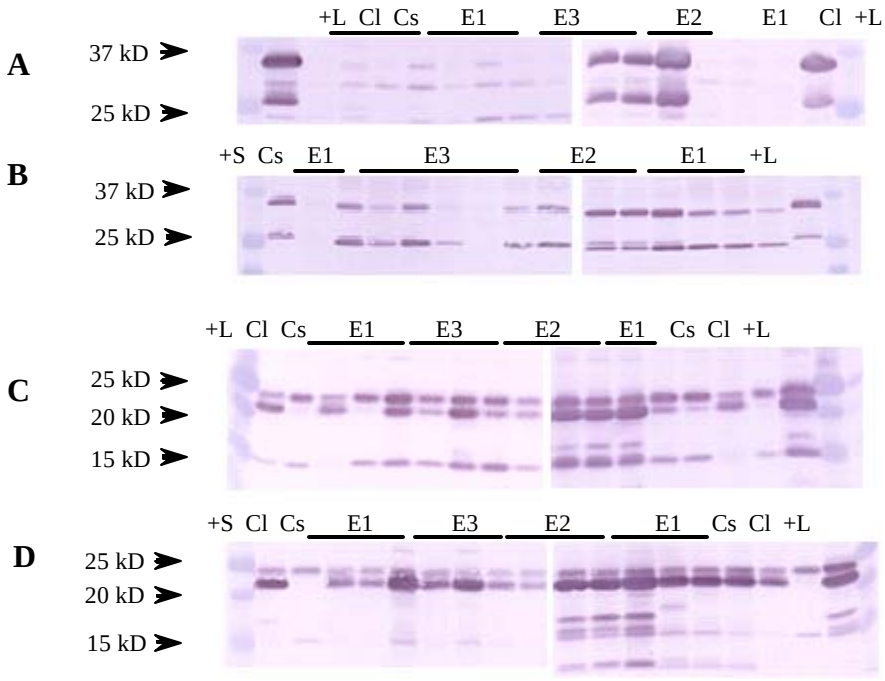

Figure 2. Western blots showing the 26-kD rice *chi* protein in (a) leaves and (b) spikes of Ever (E2) plants which was not present in Events 1 and 3 (E1, E3) transgenic and in wildtype Conlor 23-kD rice *tlp* protein was highly expressed in (c) leaves and (d) spikes of all Event 2 plants but similar band was detected in Events 1 and 3 plants. Fifteen μg of total soluble protein was loade per lane. +L = leaf, positive control; Cl = wildtype Conlon leaf; Cs = wildtype Conlon spike.

TOWARDS HIGH RESOLUTION TWO-DIMENSIONAL GEL ELECTROPHORESIS OF FHB INFECTED WHEAT SPIKE PROTEINS USING A PROTEIN FRACTIONATOR AND NARROW PH RANGE GELS

Wenchun Zhou*, François Eudes, André Laroche and Denis Gaudet

Research Centre, Agriculture and Agri-Food Canada, 5403 1st Ave. S., Lethbridge, AB T1J 4B1, Canada
*Corresponding Author: PH: (403) 317-2269; E-mail: zhouw@agr.gc.ca

OBJECTIVES

To enhance resolution of two-dimensional gel electrophoresis (2-D GE) and to improve detection of low abundant proteins in wheat spikes.

INTRODUCTION

Identification of proteins related to different phenotypes has been carried out in an extensive range of biological tissues since the mid 1970s' with the development of gel electrophoresis. Proteomics is the systematic analysis of the protein expression in a tissue, cell, or subcellular compartment. Ultimately, a proteome analysis should include most if not all the proteins from a biological sample. Two-dimensional gel electrophoresis (2-D GE) is the backbone technique of current proteomics because it enables to simultaneously separate complex mixtures of thousands of proteins that can be found in common biological samples. 2-D GE of proteins with broad range immobilized pH gradient (IPG) gel strips such as pH4-7 and pH3-10 is commonly used for displaying proteins A major setback of wide range IPGs is their limitation in visualizing less abundant proteins and in resolving proteins with either similar pI or molecular weight (Hoving et al., 2002). To enhance resolution and improve detection of low abundant proteins in wheat spikes, proteins extracted from wheat spikes were fractionated with a Zoom Fractionator prior to 2-D GE and then narrow range IPG gel strips with pH ranges matching that of each fraction of proteins were used for isoelectric focusing (IEF) before size separation.

MATERIALS AND METHODS

Plant materials - Wangshuibai, a Fusarium head blight resistant landrace from Jiangsu, P. R. China, was used in this study. Growth and inoculation of plants are described in the accompanying manuscript.

Protein extraction - Wheat spikelets inoculated with either H_2O or *Fusarium graminearum* were removed from spikes with a pair of forceps. About 15 treated spikelets from different spikes (5-8) were mixed as one sample and were ground in pre-chilled mortar with liquid nitrogen. Finely ground powder was collected into 2 ml microcentrifuge tubes and weighed. One ml of 10% (w/v) trichloroacetic acid, 0.05% (v/v) 2-mercaptoethanol in cold (-20°C) acetone was added to 0.3g of ground tissue. The samples were incubated for 2 h at -20°C to precipitate proteins and then centrifuged for 20 min at 16,000 g. The pellet of precipitated proteins was washed with 1 ml cold acetone containing 0.05% v/v 2-mercaptoethanol several times until the pellet was colorless. A 10 min centrifugation at 16,000g was used to pellet the proteins after each wash. Pellets were dried under vacuum for 10 min, and the proteins were resuspended with 1 ml of rehydration buffer (5 M urea, 2 M thiourea, 2% CHAPS, 20 mM DTT, and 0.5% carrier ampholytes pH3-10 (Invitrogen, Carlsbad, CA 92008, USA) for 1 h. After centrifugation at 16,000g for 10 min, the supernatant was collected, and a 10ml sample was removed for protein assay. The remaining supernatant was stored at -80°C until protein fractionation. Protein concentration of samples was determined using bovine serum albumin with the Bradford method (Bradford, 1976).

Protein fractionation - A Zoom® IEF Fractionator from Invitrogen (Carlsbad, CA 92008, USA) was used to fractionate isolated protein samples into different pH range fractions on the basis of isoelectric points (Zuo and Speicher, 2000). Six Zoom® polyacrylamide disks with pH values of 3.0, 4.6, 5.4, 6.2, 7.0, and 10.0 were used to form five chambers with successive pH ranges within the fractionation unit. A total of 2 mg protein of was loaded in the five chambers and separation was conducted as described in the users' manual. After fractionation, the five individual protein samples were removed from the individual chambers and stored to -80°C until loaded on IPGs gels

Protein Isoelectric Focusing and SDS-PAGE - Fractionated samples were loaded on IPGs gel strips on 11 cm pH3-10 or pH 4-7 IPGs gel strips according to the users' manual (Bio-Rad Ltd.), electrofocused and separated on small format polyacrylamide gels (10X12cm) for separation of samples based on their molecular size. On occasion, two 7 cm narrow pH range IPGs were run on large format gel (20X20 cm) to permit side by side comparison of samples. Alternatively when fractionation was omitted, 50 µg of solubilized proteins was mixed with 2 vol of rehydration buffer (6M urea, 2M thiourea, 2%CHAPS, 1% DTT and 0.5% ampholytes) and loaded on IEF gel strips according to the user's manual of the supplier. IEF was carried out according to the supplier's instruction. After IEF, the strips were either kept at -20ºC or directly used in SDS-PAGE. The strips were equilibrated in equilibration buffer I (6M Urea, 2% SDS, 0.05M Tris-HCl, (pH 8.8), 20% (w/v) glycerol, 2% (w/v) dithiothreitol) at ambient temperature for 15 min, and then in equilibration buffer II (6M Urea, 2% SDS, 0.05M Tris-HCl, (pH 8.8), 20% (w/v) glycerol, 2.5% (w/v) iodoacetamide) for another 15 min. After equilibration, the strips were positioned on top of the second-dimension gel and sealed with 1% agarose. SDS-PAGE was performed on 15% polyacrylamide gels. The small format gels were run for 1h at 200 V. The large format gels were run for 30 min at 30 mA followed by 60 mA for 6 h.

RESULTS AND DISCUSSION

Efficient protein fractionation by ZOOM® IEF Fractionator

About 2 mg protein extracted from spikes of Wangshuibai was applied on the ZOOM® IEF Fractionator. The 2D separation of fractionated samples on broad IPGs showed that wheat spikes contain a complex combination of polypeptides (Fig. 1A). Results showed that the ZOOM® IEF Fractionator was able to effectively separate this very complex mixture of proteins into several pH ranges as showed in Fig. 1B-E. Reproducible separation of complex protein samples into distinct liquid fractions was realized using this instrument. One disadvantage of ZOOM® IEF Fractionator apparent in our experiments was a protein loss of 40% during fractionation. This was likely due to an entangling of proteins in the polyacrylamide disks separating the different chambers.

Enrichment of low abundant proteins on narrow pH IPGs gel strips

The fractionated protein samples from Wangshuibai spikes were loaded on 7 cm IPGs gel strips with narrow pH ranges that were 0.1 unit wider than the pH ranges of each fraction at both ends. For example, 7 cm IPGs with pH range of 6.1 to 7.1 were used to load proteins in the fraction of pH 6.2 to 7.0, and two 7 cm IPGs were run on 20x20 cm PAGE in the second dimension (Fig.1F-G). Fractionation of protein samples using the ZOOM® IEF Fractionator enables enrichment for less abundant polypeptides (eg. spot #2 in Fig. 1F) thereby making them visually detectable. Spot 2 was not visible when 200 µg of whole protein sample was directly loaded on the same 11 cm IPG gel strips during the 1st dimension separation (Fig. 1A). The fractionation protocol permitted the identification of differentially expressed proteins between FHB infected and H_2O inoculated spikes such as spots #1 and 2 in Fig.1F-G.

In conclusion, the ZOOM® IEF Fractionator can separate wheat spike proteins into several fractions of different pH ranges. Narrow pH ranges IPGs matched with all fractions were available for 2D-GE analysis. Higher resolution of protein profiling, enrichment for less abundant proteins and identification of differentially regulated proteins were achieved in this experiment.

ACKNOWLEDGEMENT

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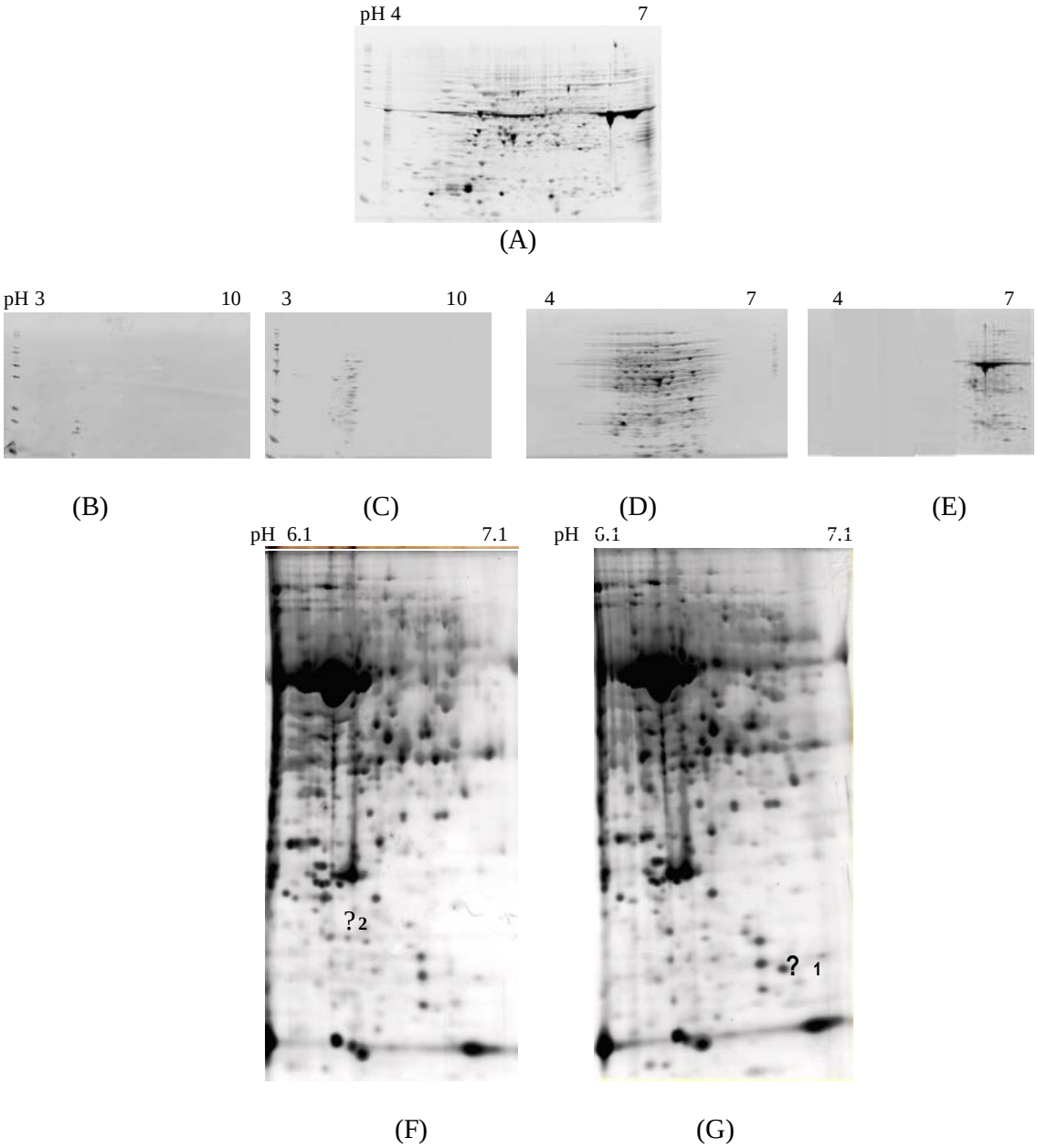

Figure 1: Two dimensional electrophoregrams of wheat spike protein samples. **A:** a whole protein extract (200 µg) separated directly on a 11 cm pH4-7 IPG gel strip in the first dimension; **B:** fractionated sample (10 µg) from pH3-4.6 range separated on a 11 cm pH3-10 IPG gel strips; **C:** fractionated sample (50 µg) from pH4.6-5.4 range separated on a 11 cm pH3-10 IPG gel strips; **D**: fractionated sample (50 µg) from pH5.4-6.2 range separated on a 11 cm pH4-7 IPG gel strips; **E:** fractionated sample (50 µg) from pH6.2-7.0 range separated on a 11 cm pH4-7 IPG gel strips; **F:** fractionated protein (50 µg) from Wangshuibai spikes 3 days after control inoculation from pH6.2-7.0 range separated on a 7 cm pH6.1-7.1 IPG gel strip. **G:** fractionated protein (50 ug) from Wangshuibai spikes 3 days after inoculation with FHB pH6.2-7.0 range separated on a 7 cm pH 6.1-7.1 IPG gel strip. Arrow 1 indicates a protein only present on FHB infected spikes. Arrow 2 indicates one protein present only in H_2O inoculated spikes. Gels A to E were stained with SYPRO-Ruby while gels F and G were stained with silver

TWO-DIMENSION DIFFERENTIAL DISPLAY OF PROTEINS ISOLATED FROM FHB INFECTED AND HEALTHY SPIKES OF WANGSHUIBAI

Wenchun Zhou*, François Eudes, André Laroche and Denis Gaudet

Research Centre, Agriculture and Agri-Food Canada, 5403 1st Ave. S., Lethbridge, AB T1J 4B1, Canada
*Corresponding Author: PH: (403) 317-2269; E-mail: zhouw@agr.gc.ca

OBJECTIVES

To identify proteins responsive to FHB infection from spikes of Wangshuibai, a FHB resistant landrace from China.

INTRODUCTION

Fusarium head blight (FHB) or scab, caused by *Fusarium graminearum* Schwabe, and sometimes by other *Fusarium* species, is a severe disease of *Triticum spp*. and *Hordeum vulgare*, causing significant reductions in yield and quality in many wheat production regions around the world (McMullen et al., 1997). Although the genetics of FHB resistance have been well documented and resistant cereal cultivars have been developed to minimize FHB impact, there is a limited understanding of the molecular mechanisms involved in plant resistance against the infection and spread of *Fusarium graminearum*.

Proteomics techniques provide an important tool to study mechanisms of plant resistance against biotic and abiotic stress. Using two-dimensional electrophoresis (2D-GE) and proteomics techniques, specific proteins have been shown to be differentially expressed in salt- and heat-stressed wheat (Majoul et al., 2000, 2003; Ouerghi et al., 2000). One of the major advantages of this technique is that differentially expressed proteins can clearly and reproducibly be detected between sensitive vs. tolerant lines, or between infected (stressed) vs. uninfected (non-stressed) conditions. Proteins that are qualitatively or quantitatively different in their expression levels among treatments have a high likelihood of playing an important role in the response of the plant to a given stress. Further identification of these differentially expressed proteins by LC-MS/MS can provide powerful insight into the molecular mechanisms of resistance and underlying functions of these proteins in determining resistance or tolerance in plants.

MATERIALS AND METHODS

Plant materials - Wangshuibai, a Fusarium head blight resistant landrace from Jiangsu, P. R. China, was used in this study. Seeds of Wangshuibai were germinated in plastic trays filled with vermiculite. After seedlings emerged, plants were transferred to a vernalization chamber for 8 weeks at 4°C with a 16 hr photoperiod. Vernalized plants were transplanted into 15 cm pots and grown in a greenhouse. About 30 pots were placed randomly on a bench in a greenhouse maintained at 24°C with a 16 hr photoperiod (artificial lights were used to maintain light intensity over 300 watts/m^2 when it is necessary). The same water and fertilizer management were used for all materials during the entire growing period.

Wheat spikelets were inoculated with *F. graminarium* conidiospores or deionized water using two syringes on the morning when they were at the mid-anthesis developmental stage. About 1000 conidiospores in a volume of 10 µl were injected into two flowering florets of a spikelet. The same volume of deionized water was injected into flowering spikelets on a different plant to serve as a control. The inoculated spikelets were marked and the time and date of inoculation recorded. Inoculated plants were placed into a mist room immediately after inoculation. The humidity in the mist room was maintained at 90% using a computer controlled high-pressure mist system. The temperature in the mist room was 24 °C and with the same light intensity as in the growth room. Following inoculation, spikes were harvested by cutting with a pair of scissors 24 h, 48h and 72 h after inoculation Harvested

spikes were immediately placed on ice, and were transferred into a -80°C freezer for storage until protein extraction.

Protein extraction and quantitation - Standard protein extraction and quantification methods described in the accompanying manuscript in these Proceedings were employed.

Isoelectric focusing and SDS-PAGE - A solubilised protein sample (150 -500µg) was mixed with rehydration buffer from Bio-Rad (Hercules, CA, USA) to a total volume of 350 µl was loaded and focused on 17 cm Bio-Rad Ready Gel Strips as described by the manufacturer's manual. For the second dimension separation, the strips were positioned on top of the second-dimension gel and sealed with 1% agarose. SDS-PAGE was performed on 15% polyacrylamide gels. The gels were run for 30 min at 30 mA followed by 60 mA for 6 h. Sample separation was repeated three times.

Staining of PAGE gels - Three staining methods were used in this experiment. The silver stainning method was used for analytical purpose. The SYPRO Ruby stain method was used for quantitative analysis. For preparative gels, Colloidal Coomassie Blue (CBB) G-250 was used. Induced and differentially regulated protein spots were excised from CBB stained gels for LC-MS/MS analysis.

RESULTS AND DISCUSSION

2-DE display of proteins from spikes -

The separation of protein samples from the spikes of Wangshuibai that were harvested 1-, 2-, and 3-days after inoculation with *F. graminearum* or water are shown on Fig. 1. Acidic proteins were displayed on immobilized pH gradient (IPG) gel strips pH range 4-7, while basic proteins were displayed on IPG gel strips pH range 7-10. Collectively, these results represented proteins within pH range 4-10 that were extracted from wheat spikes. Analyses of results showed that under our conditions the 2-DE technique used was highly reproducible for proteins isolated from both FHB infected and healthy spikes.

Proteins displayed differentially between healthy and FHB infected spikes - Both qualitative and quantitative differences of protein expression were observed between healthy and FHB infected spikes on 3 time courses. In total, twelve protein spots ranging from 6 to 120 kilodaltons were detected only in proteins from FHB infected spikes. For example, two different protein spots from isolated spikes sampled 2- and 3-days after inoculation with FHB are shown in Fig. 2. More than twenty spots ranging in molecular size from 6 to 120 kilodaltons were also detected either as being up- or down-regulated following inoculation with FHB infection. These protein spots have been excised and we are waiting for the LC-MS/MS results to identify all these proteins with altered levels of expression caused by FHB infection.

The utilization of 2D-GE has enabled the reproducible identification of differentially regulated polypeptides and mass spectrophotometry results will permit protein identification whether these proteins originate from wheat or the pathogen and provide an indication of which biochemical pathways are involved following infection of the FHB wheat land race Wangshuibai.

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1- (A, B), 2-(C, D) and 3-day (E, F) after inoculation with *Fusarium graminearum* (B, D, F)or water (A, C, E). The small dashed box on 3-day after inoculation indicates the close-up region shown in Figure 2.

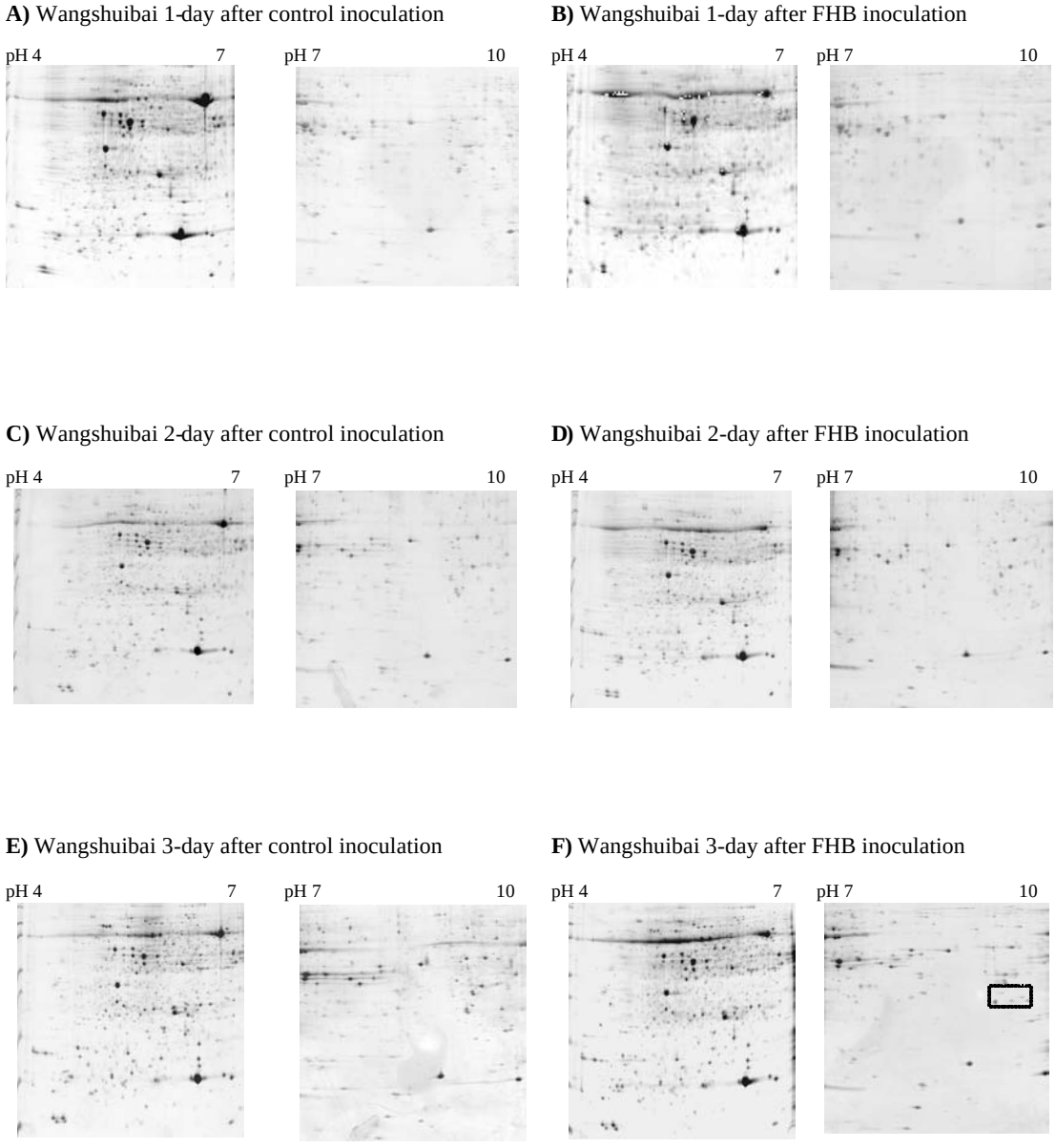

Figure 1. Two dimensional electrophoregrams of protein samples isolated from Wangshuibai wheat spike harvested

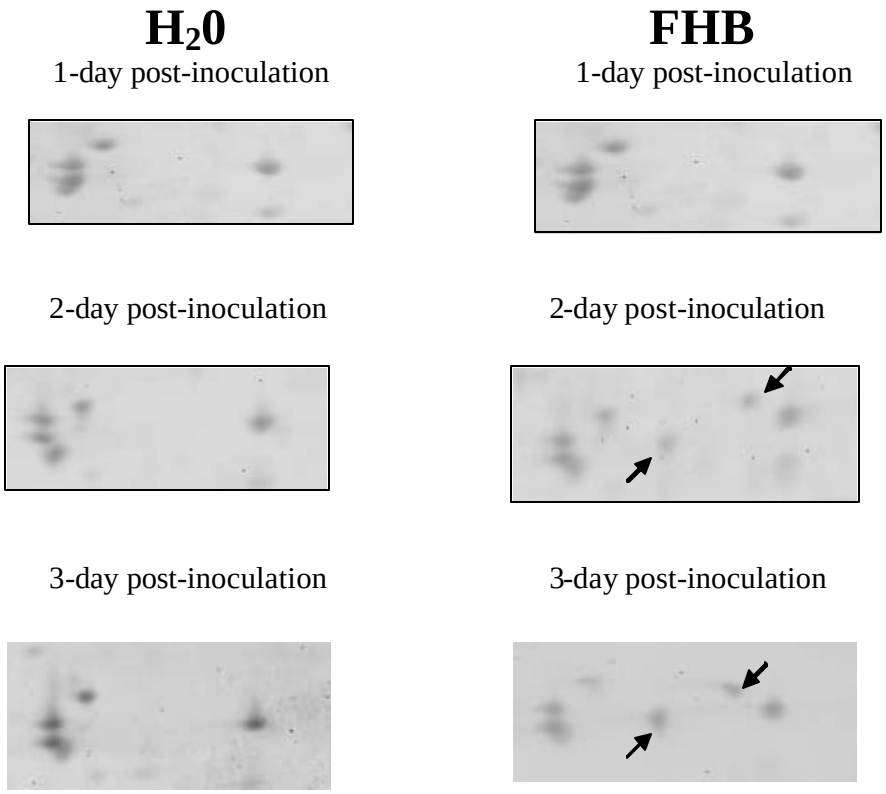

Figure 2. A close-up comparison of expression of some basic proteins in FHB inoculated and control spikes. Arrows point at spots that were only shown in protein samples isolated from spikes harvested 2- and 3-day after inoculation with *Fusarium graminearum.*