INTRODUCTION OF A MODIFIED RIBOSOMAL PROTEIN L3 GENE AS A STRATEGY TO INCREASE TRICHOTHECENE TOXIN RESISTANCE IN PLANTS

Gerhard Adam*, Rudolf Mitterbauer, Armin Raditschnig,
Hanna Weindorfer, and Josef Glössl

University of Agricultural Sciences, Center of Applied Genetics, Muthgasse 18/05/66, A-1190 Vienna, Austria.
*Corresponding author: PH: (43) 1-36006-6380, E-mail: adam@edv2.boku.ac.at

OBJECTIVES

The goal of our project is to identify alterations in the ribosomal target of trichothecenes, the ribosomal protein L3, which confer toxin resistance, and to explore the feasibility of increasing toxin resistance in transgenic plants by expression of engineered RPL3 genes.

INTRODUCTION

Trichothecene myctoxins act primarily as inhibitors of eukaryotic protein synthesis. Gene disruption studies indicate that production of deoxynivalenol (DON) is a virulence factor of Fusarium graminearum. The probable role of the toxin is to inhibit active defense responses of the plant by interfering with the expression of defense related proteins and to condition the host for colonization. Correlative evidence is available that toxin resistance significantly contributes to field resistance of wheat. We used yeast as a model system to explore molecular mechanisms responsible for trichothecene resistance and identified semidominant mutations in the gene encoding ribosomal protein L3. Transgenic tobacco was used as a model to test the feasibility of increasing toxin resistance in plants by introducing a modified copy of RPL3.

MATERIALS AND METHODS

In order to save expensive toxin, all yeast strains employed contain a disruption of the gene PDR5, which encodes a plasma membrane localized ABC transporter protein with specificity for trichothecenes (Adam & Lemmens, 1996). For the shuttle mutagenesis of yeast RPL3 a strain was constructed containing a deletion of the chromosomal RPL3 gene. This strain (YZGA315) is viable on galactose medium due to a plasmid allowing expression of RPL3 under control of the glucose repressible GAL1 promoter. A plasmid, which contains a wildtype copy of RPL3 and the TRP1 and ADE2 genes as selectable markers, was mutagenized by passage in the E. coli mutator strain XL1-Red (Stratagene) and by hydroxylamine-mutagenesis. Upon transformation of YZGA315, colonies were selected on glucose based medium and transferred to plates containing 100 ppm DON. Plasmid was recovered from resistant colonies, and the alterations responsible for resistance identified by subcloning and sequencing.

A tomato cDNA clone LeRPL3 was isolated from a phage lambda cDNA library and sequenced. One of the alterations identified in yeast was introduced into the gene by overlap

extension PCR. Furthermore, a c-Myc epitope was added at the C-terminus. The constructs were cloned into a binary plasmid behind the 35S promoter and introduced into tobacco by Agrobacterium-mediated transformation. Transgenic plants were characterized by Southern and Western blotting and tested for alterations in toxin resistance using seed germination, leaf disk regeneration and a gravitropism assay.

RESULTS AND DISCUSSION

Semidominant mutations in RPL3 (formerly known as TCM1) conferring resistance to the trichothecene trichodermin had been described previously (Fried & Warner, 1981), but the nature of the mutation was not determined. We have performed a random mutagenesis of yeast RPL3 and characterized 100 plasmids conferring resistance to DON. Five single amino-acid alterations in four different positions of the protein which lead to DON resistance were identified by subcloning and sequencing. In addition, we have also identified the amino-acid change in two of the original TCM1 mutants. One of the changes (W255C in ScRPL3) was engineered into the highly homologous tomato RPL3 cDNA and introduced into yeast and tobacco.

The tomato gene, without and with a C-terminal epitope tag is able to complement a yeast mutant containing a deletion of RPL3. In addition, the engineered version of the tomato cDNA also confers DON resistance in yeast. Subsequently we have introduced the wildtype and the engineered versions of LeRPL3, with and without the c-Myc tag into tobacco.

Characterization of the several transgenic plants in different assays (seed germination on toxin containing medium, regeneration of leaf disks, gravitropism response in the presence of DON) revealed that, at best, a disappointingly small increase in toxin resistance could be achieved. Analysis of protein extracts of the transgenic plants furthermore showed that, in contrast to the wild type protein, only traces of the tagged Rpl3p containing the mutation are detected. Our hypothesis is that the mutant RPL3 protein has a disadvantage during assembly into the ribosome, and that this protein is rapidly degraded. A similar phenomenon was observed in yeast heterozygous for RPL3wt/RPL3W255C. Despite being present in unstressed yeast in a low amount, the mutant form of the protein confers semidominant resistance. Pretreatment of yeast with sub-inhibitory amounts of DON leads to a rapid accumulation of the mutant form of the protein and dramatically improved toxin resistance.

In summary, we have identified several mutations in the ribosmal target that could become potentially useful in the screening of wheat germplasm for natural ribosomal resistance (Miller & Ewen, 1998) and for biotechnological approaches. Yet, our results also suggest that the prospects to achieve a marked increase in toxin resistance by simply introducing a modified RPL3 gene into wheat are not very good. Wheat contains most likely six RPL3 genes contributing to the pool of Rpl3p – a highly competitive situation.

We can only speculate whether or not an engineered Rpl3 protein - or a naturally existing variant conferring higher resistance - would accumulate in wheat tissue (with rapid synthesis of ribosomes), to give biologically meaningful resistance, as in the case of the unicellular yeast model. It is conceivable that such an effect takes place in the low concentration range of the gradient of toxin diffusing ahead of the fungus.

In an optimistic scenario, this could still be sufficient to allow adaptation, defense gene expression and containment of the spreading fungus, without leading to a prominent resistance phenotype in in vitro assays based on challenge with toxin levels blocking protein synthesis immediately. Further research is needed to answer this question.

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REDUCED VIRULENCE OF FUSARIUM GRAMINEARUM MUTANTS DEFICIENT IN TRI101: TRANSACETYLASE ACTIVITY

N.J. Alexander*, S. P. McCormick, and S.L. Ziegenhorn

USDA-ARS, National Center for Agricultural Utilization Research, Peoria, IL, 61604
*Corresponding author: PH: (309) 681-6295; E-mail: alexannj@mail.ncaur.usda.gov

ABSTRACT

Fusarium graminearum, the causative agent of wheat head blight, produces the trichothecene deoxynivalenol (DON). DON acts as a protein synthesis inhibitor and is a significant factor contributing to the disease. However, acetylation of the C-3 hydroxyl group of a number of different trichothecenes reduces the toxicity to yeast as well as to the single-celled plant, Chlamydomonas, suggesting that the C-3 OH site is critical for toxicity. Since the TRI101 gene in F. graminearum encodes a 3-O-acetyltransferase, this gene may function for self-protection against DON and its intermediates. Disruption of the FgTRI101 was done to test if, and to what extent, this gene is involved in self-protection. The degree of virulence on wheat by mutants deficient in TRI101 activity has also been evaluated. FgTRI101 was cloned from Gz3639 into the vector pT7Blue3. The selectable marker, hygromycin B, as well as its promoter, was inserted into an EcoRI site, located approximately in the middle of the 1.4 kb DNA sequence encoding FgTRI101. Disruption was verified by both PCR and Southern hybridization. Fungal disruptants were tested on toxin-containing medium to determine if the mutants were more sensitive than the wild type strain. They were also analyzed to determine what intermediates in the pathway they synthesized. Previous work in our labs has shown that F. sporotrichioides mutants carrying a disrupted FsTRI101 gene accumulate isotrichodermol, a compound shown by us to be toxic to Chlamydomonas. However, TRI101-disrupted mutants in F. graminearum do not accumulate the C-3 hydroxylated compound and instead accumulate the C-3,8 hydroxylated compound, which has been shown to be less toxic in our Chlamydomonas toxicity tests. To test whether FgTRI101 disruptants were less virulent than wild type, wheat virulence testing in the greenhouse showed that the fungal TRI101 disruptants produced far less disease than the wild type. Our results support the conclusion, that in F. graminearum, FgTRI101 plays a role in self-protection but is not the sole mechanism. Disruption mutants are weakened in their ability to infect and cause disease in wheat, most likely because they do not produce large quantities of a C-3 OH toxic byproduct.

CHARACTERIZATION OF WHEAT PR-PROTEINS CDNA'S FOR TRANSFORMATION OF WHEAT TO ENHANCE RESISTANCE TO SCAB

A. Anand[1], W. Li[2], N. Sakthivel[1], S. Krishnaveni[1], S. Muthukrishnan[1]*, B.S. Gill2, J.S.Essig[2], R.E.Adams[2,] V.Janakiraman[2], H.N.Trick[2]

[1]Department of Biochemistry; [2] Department of Plant Pathology,
Kansas State University, Manhattan, KS, 66506
*Corresponding author: PH: (785) 532-6939, E-mail: smk@ksu.edu

ABSTRACT

Chitinases and b-1,3-glucanases are important components of plant defense in response to attack by pathogens. To identify specific chitinases and b-1,3-glucanases involved in plant defense, a cDNA library was constructed using mRNA from wheat spikelets inoculated with conidia of Fusarium graminearum. Two chitinase and two b-1,3-glucanase clones were isolated using a rice class I chitinase and barley class II chitinase cDNA clone and a barley b-1,3-glucanase as probes. Northern blot hybridization showed that the expression of these genes is induced upon infection with Fusarium graminearum. The accumulation of transcripts for these PR-proteins is more rapid in the resistant variety Sumai 3 than its susceptible mutant during the first 24 hrs after infection. Spring wheat, 'Bobwhite', a scab-susceptible cultivar was transformed with pAHC20 vectors carrying the bar gene and the gene of interest under the control of maize ubiquitin promoter-intron. The primary transgenic lines were characterized based on PCR detection for bar gene, gene(s) of interest and western blot analyses. The integration and inheritance of the transgene and the bar gene was detected in the T1 progenies based on Southern hybridization with target gene and PCR analyses. Liberty (0.1%) painting in the young leaves confirmed the expression of the bar gene and the western blot analysis confirmed stable expression of the transgene. Several transgenic lines containing single or different combinations of PR-proteins have been identified and are being propagated.

INTRODUCTION

Plants express a wide variety of genes in response to pathogen/pest infection. Such genes are referred to as pathogenesis-related (PR) genes (Bowles 1990). The best characterized genes belonging to this group are those that encode the hydrolytic enzymes, chitinases (EC 3.2.1.14) and b-1,3-glucanases (EC 3.2.1.39). These hydrolytic enzymes inhibit the growth of many fungi in vitro by hydrolyzing chitin and b-glucan of fungal cell walls and the digestion products of chitin and b-glucan can act as signal molecules to stimulate further defense responses. Wheat scab, caused by Fusarium graminearum, can be a devastating disease that not only lower grain yield, but also adversely affects the grain quality as well, as a result of the accumulation of toxins. A cDNA library of mRNA isolated from scab-infected 'Sumai 3' a scab-resistant variety was constructed, and cDNA clones encoding chitinases and glucanases were isolated using rice and barley clones for chitinase and b-1,3-glucanase as probes.

The advancements and refinement in the plant transformation protocol for monocot crops including wheat (Zhang et al. 1999; Chen et al. 1999; Gaunt et al. 1999) have increased the efficiency of introducing foreign and agronomically important genes into wheat. Naturally occurring antifugunal genes (PR-proteins) from plants and other microrganisms are documented to enhance the level of disease resistance in wheat (Chen et al. 1999; Clausen et al. 2000). The application of plant transformation protocol to introduce environment-friendly defense gene(s) into crop plants is preferable to unsustained use of fungicides to control wheat scab. In this report we document the transfer and constitutive expression of wheat defense genes isolated and characterized from a scab-resistant cultivar for scab resistance in wheat.

MATERIALS AND METHODS

Isolation, mapping and characterization of the PR-proteins

GZ3639, a highly virulent isolate of F. graminearum, was used at a final concentration of 1.8x105 spores ml-1 to inoculate Sumai 3 (c'Funo' x 'Taiwan Wheat') a scab resistant cultivar and its mutant susceptible variety. The cDNA library construction and screening was described by Li et al. (2000). The first 60 RILs of the ITMI population and 56 F2 plants of the Ae. tauschii population were used for genetic mapping. Base maps were constructed (Li et al. 1999) for placement of chitinase and glucanase genes. The positive clones were cloned in the T- vector and the plasmid DNA from these were used as templates for sequencing. Open reading frames (ORF's) and amino acid sequences were deduced using the ORF Finder program, and homology searches were conducted using the BLAST 2.0 program of the National Center of Biotechnology Information (NCBI) at the website http://www.ncbi.nlm.nih.gov. The theoretical isoeletrical points, molecular weights, and cleavage sites of signal peptides were analyzed with "DNA & Protein Analysis Toolkit" at the website http://www.rockefeller.edu/rucs/toolkit/toolkit.html.

Wheat transformation

The plant transformation vectors harboring the wheat chitinase(s) and b-1,3-glucanase(s) genes were generated by ligating the chitinase and glucanase coding region with a maize ubiquitin promoter-intron DNA and the linearized pAHC20 vector (Christensen et al. 1992) with the selectable marker, bar gene. Immature embryos of the spring wheat cultivar, 'Bobwhite', were co-transformed with these vectors using the particle inflow gene gun. The DNA's were coated on tungsten particles and different combinations of defense genes were employed in the co-transformation experiments. The primary transformants were selected on glufosinate plates (5 mg/l) and the regenerated T0 plants were subjected to PCR detection for bar gene and the gene(s) of interest using gene specific primers. Western blot analyses were carried out using appropriate antisera. Approximately 12.5 mg total genomic DNA from each T1 plant was digested with Hind III (80 U) and probed with 32P-labelled bar gene and the transgene fragments by Southern hybridization. Liberty [0.1% (w/v)] painting was carried out on the young leaves for confirming the stable expression of the bar gene in the T1 plants.

RESULTS AND DISCUSSION

Four clones, SM169, SM194, SM233 and SM383 were identified when 400 clones of the cDNA library were screened using a mixture of the inserts of clones Chi11 (rice) and HvChtN12 (rice) as probes. SM194 and SM383 contain cDNA inserts of 956 bp and 1088 bp, respectively. SM194 and SM383 are similar to SM169 and SM233 in insert sizes, respectively. Two cDNA clones, SM289 and SM638, were isolated by screening 880 clones of the cDNA library using the barley b-1,3-glucanase cDNA clone BH72-I1 (barley) as the probe. The cDNA inserts in SM289 and SM638 are 1269 bp and 1439 bp, respectively. The details of these clones are presented in Table 1.

Southern analysis of nullitetrasomic (NT) lines with cDNA inserts of the chitinase clones, SM194 and SM383, gave identical hybridization patterns and produced about 10 bands (EcoRI digest). All the bands were localized to group 2 chromosomes of wheat. Four polymorphic and two monomorphic bands were detected by SM194 and SM383 in the Ae. tauschii parents. They were mapped to the long arm of chromosome 2D at a position between markers Xwg405 and Xpsr102, which appear to be genetically close to the centromere (Boyko et al. 1999).

Analysis of NT lines assigned the b-1,3-glucanase genes, SM289 and SM638, to group 3 chromosomes just as BH72-I1, the probe used for their isolation. Genetic mapping localized SM289 to 3BL and 3DL (Xksu933 (Glb3) and SM638 to 3DL (Xkus934 (Glb3) of wheat. These loci map very close to the position of b-1,3-glucanase loci (Glb3) detected by BH72-I1 (Li et al 1999). This result not only confirmed the presence of a b-1,3-glucanase gene cluster in 3BL, but also revealed another cluster of b-1,3-glucanase genes in 3DL of wheat.

Table1: Characteristic properties of the wheat cDNA clones

Clone name	Type of PR-protein	Size of cDNA insert (bp)	Size of polypeptide (ORF)	Molecular weight
SM194	Chitinase (type VII)	956	230 AA	24.7 kDa
SM383	Chitinase (type IV)	1082	272 AA	29.03 kDa
SM289	β-1,3-glucanase	1269	334 AA	34.88 kDa
SM638	β-1,3-glucanase	1439	334 AA	34.66 kDa

Northern blot hybridization showed that the expression of the chitinase and b-1,3-glucanase genes characterized in this study is induced in wheat spikelets upon infection with F. graminearum. The transcription profiles of these genes were different in the scab-resistant cultivar Sumai 3 and the scab-susceptible mutant indicating their role in disease resistance. The expression levels at three time points studied follow the pattern: 24 hai³48 hai>72 hai in Sumai 3, but in the mutant, the pattern generally is 24 hai<48 hai>72 hai. The transcripts of both chitinase and b-1,3-glucanase genes reached the maximum point at or before 24 hai in Sumai 3 while in the mutant, the peak values were not reached until 48 hai or later indicating a slower defense response in the mutant (Fig. 1). For SM194, a larger transcript was detected clearly at 24 hai in Sumai 3, but it was still very weak at 48 hai in the mutant. This messenger might be transcribed from another member of class VII chitinase genes. These results suggest that the mutant may be defective in a common step leading to the induction of both chitinases and b-1,3-glucanases and they are in agreement with results reported recently by Pritsch et al. (2000) who showed that several PR-protein genes were induced earlier in Sumai 3 than the susceptible (non-isogenic) genotype, 'Wheaton'.

Bobwhite embryo's were bombarded with tungsten particle coated with plasmid DNA's harboring the wheat chitinase (pAHC 194; 383) and b-1,3-glucanase (pAHC 289; 638) singly and in combination with the ubiquitin promoter driving the expression of both the selectable marker and the gene(s) of interest. The bombarded embryogenic calli were grown on glufosinate at 5 mg/l and the selection pressure was maintained till plants were regenerated. In all, 38 primary transgenic lines were raised and they were subjected to PCR analyses for the presence of bar gene. Twenty-three of the bar positive lines also showed the presence for the gene of interest by PCR. The results from five lines are shown in Fig. 2. Protein extracts from these lines reacted with appropriate antisera for chitinase and glucanase and the expected protein bands of 26.5 or 29 kDa was detected in western blots. However, the level of expression varied among different events as shown in the Fig. 3. Some lines expressed both the chitinase and the b-1,3-glucanase (lines #32, #44 and #58).

Only eight of the 23 lines were included in the current analyses, the analyses of the remaining 15 lines are in progress. Five seeds were germinated from each independent line for raising the T1 progeny. All the T1 progenies were painted with 0.1% Liberty at the stem elongation stage and checked for Liberty-resistance. The sensitive plants showed browning of tissues and clearing of chlorophyll in the painted areas of the leaf within five days, while the painted areas of the resistant lines remained green. In all, 4 out of the 8 primary transgenic lines tested were Liberty- resistant.

The inheritance of the intact transgenes was confirmed by PCR for bar gene and the gene(s) of interest in the T1 progeny. The total genomic DNA from 5 different T1 plants representative of each transgenic line were digested with the HindIII enzyme which is expected to release the 3.1-3.3 kb fragment of the intact coding region of the transgene plus the promoter. Non-transgenic and Liberty-sensitive plant was used as the negative control. In the Southern blots, DNA from transgenic plants had the expected band for bar, 383 chitinase, and 638 glucanase genes when probed with the 570 bp fragment coding region of bar gene, or the 1.1-1.3 kb fragments containing the intact coding region of the 383 and 638 transgenes respectively (Fig. 4a&b). DNA from the non-transgenic and Liberty-sensitive plants exhibited no hybridization to bar coding region fragment. No bands of 3.1-3.3 kb

size expected for the intact trasngene was detected in the non-transgenic controls, even though other bands, presumably wheat chitinase and b-1,3- glucanase genes, were detected in all lines including non-transgenic controls.

These four lines were further assayed for the stable expression chitinase and glucanase. Crude protein extracts from the 5 plants each from the representative lines showed differences in the level of expression, with a few PCR and Southern positive lines showing comparable levels of transgene expression in the T1 progeny. The highest expression levels were seen in plants T1-32-1, 32-2 and 26-5. The line 26-5 which is a singly-bombarded line with the 383 chitinase gene had the expected 26.5-kDa protein band that reacted with a barley chitinase antibody. The lines 32-1 and 32-2 that were cobombarded with the 383 chitinase and 638 glucanase genes showed strong reaction with the chitinase and glucanase antibodies and gave the expected protein products of 26.5 and 29 kDa. This confirmed the stable and constitutive expression of the transgenes in these progenies. The T2 progenies of these lines will be assayed further and bioassay with the test pathogen will be done to confirm whether these genes have enhanced the level of resistance in wheat.

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Fig.1. Northern blot for the expression of chitinase (SM194 and SM383) and the β-1,3- glucanase (SM289 and SM638) in the scab-resistant variety Sumai 3 and its susceptible mutant.

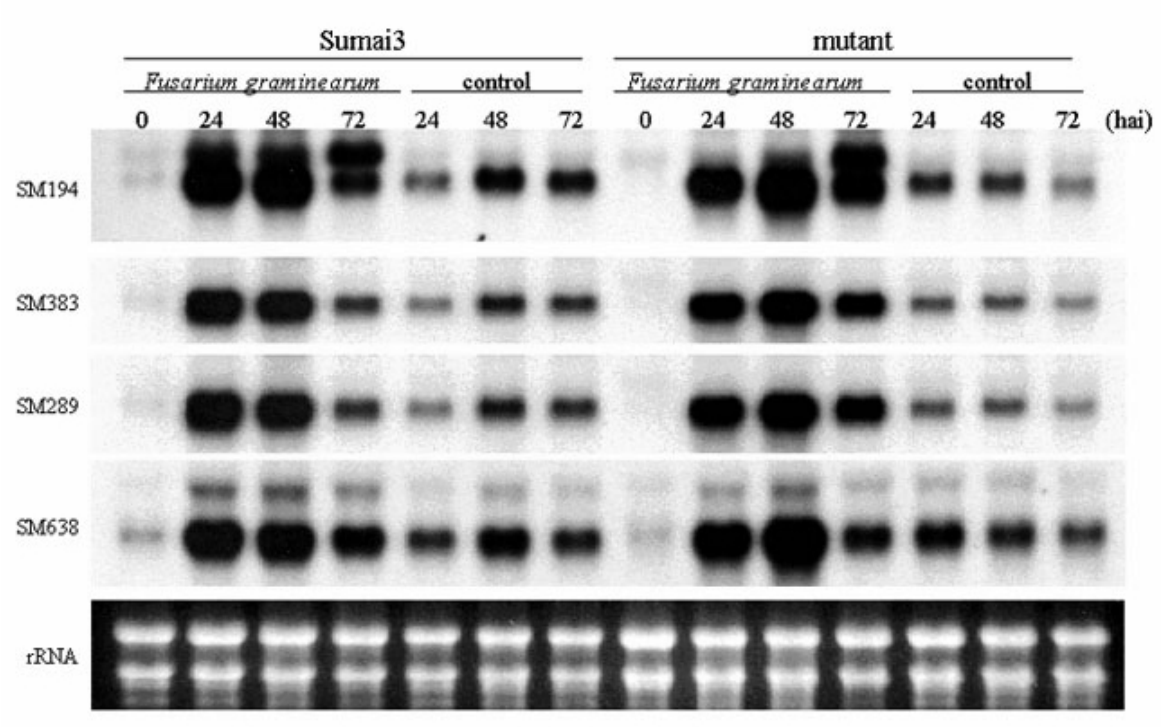
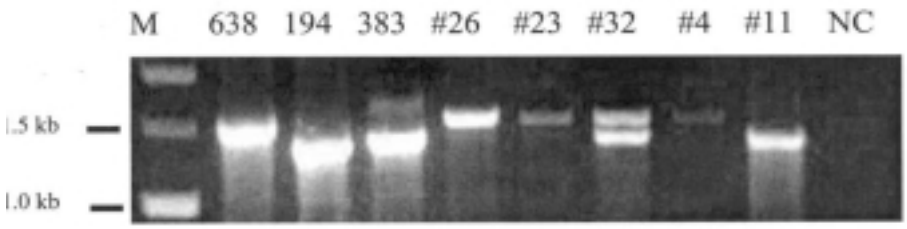
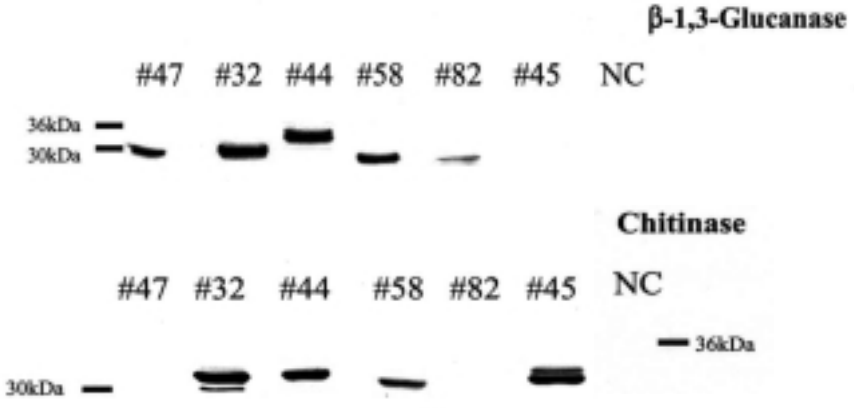


Fig. 2. PCR detection of the transgene(s) in the primary transgenic lines



#32: cobombarded with 2 gene, #26, #23 #4 & #11: with single gene insert; 638; 194; 383: plasmid controls;NC: non-transgenic plant

Fig. 3. Western blot of the putative transgenic lines expressing different PR-proteins

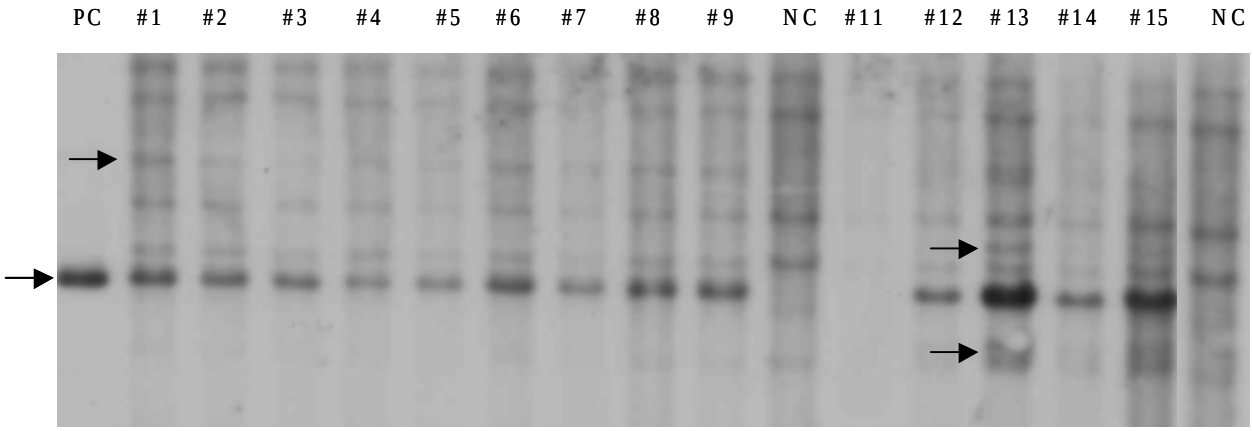


#47, #32, #58: 383:638; #82: 638:194; #45:194:383 ;#44: 289:383; NC: non-transgenic plant

194, 383: wheat chitinase; 289, 638: wheat glucanase

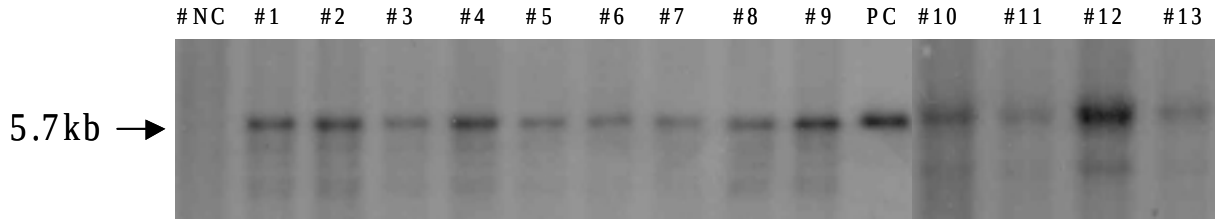
Fig. 4. Southern blot analysis of DNA from T_1 progenies of plants # 32 and # 26

a) DNA blot hybridized with 383 chitinase probe



PC: plasmid control, #1to #9: progenies of line 32, #11 to #15: progenies of line 26 and NC: non-transgenic plant. Arrows indicate the bands due to transgene.

b) DNA blot hybridized with *bar* probe



PC: plasmid control, # to #9: progenies of line 32, #10 to #13: progenies of line 26 and NC: non-transgenic plant.

MOLECULAR MAPPING OF A QTL FOR DEOXYNIVALENOL TOLERANCE IN WHEAT

[1]G-H Bai, [2]R. Plattner, [3]G. Shaner and [4]F. Kolb*

[1]Oklahoma State University, Stillwater, OK; [2]NCAUR, USDA/ARS, Peoria, IL;
[3]Purdue University, West Lafayette, IN, University of Illinois, Urbana, IL, 61801
*Corresponding author: PH: (217) 333-9485, E-mail: f-kolb@uiuc.edu

ABSTRACT

Deoxynivalenol (DON, a mycotoxin) produced by *Fusarium graminearum* in wheat grain is detrimental to livestock and humans. Significant differences in DON levels of infected grain are observed among wheat cultivars after infection with the fungus. However, genetic control of DON accumulation in wheat is not well characterized. To map quantitative trait loci (QTL) for low DON level in infected wheat grain, an amplified fragment length polymorphism linkage map was constructed using F9 recombinant inbred lines (RILs). The mapping population was derived from a cross between cultivars Ning 7840 (7 ppm of DON) and Clark (151 ppm of DON). Flowering spikes of F_{11} RILs were sprayed with conidia of *F. graminearum* in the greenhouse. The inoculated spikes were harvested at maturity, and the kernels were analyzed for DON levels with a fluorometric quantification method. One QTL for low DON level was identified, which explained about 23 and 26% of the phenotypic variance in two repeated experiments, respectively.

INTRODUCTION

Scab of wheat, mainly caused by *Fusarium graminearum*, can dramatically reduce grain yield and quality. Infected grain is often contaminated with deoxynivalenol (DON), a potent mycotoxin produced by *F. graminearum*. Because DON contamination in infected grain is a major concern for human and animal health, scab has become an especially serious disease in many wheat and barley production areas.

DON levels in harvested grain can vary greatly among wheat cultivars, ranging from a trace to more than 1000 ppm depending on wheat cultivar and environment. However, the genetic control of DON production by *Fusarium* species in wheat grain is largely unknown. Ning 7840 is a highly scab-resistant spring wheat cultivar with low DON, and Clark is a highly susceptible winter wheat cultivar with a high level of DON accumulation in infected grain. One major QTL for Type II scab resistance has been identified (Bai, et al, 1999). In this study, we use an AFLP map to identify QTL for low DON level in scab-resistant wheat cultivar Ning 7840.

MATERIALS AND METHODS

Wheat cultivars Ning 7840 (low DON) and Clark (high DON) as well as their 133 recombinant inbred progenies (Bai et al, 1999) were inoculated by spraying conidia (500 spores / mL) of a DON-producing strain of *F. graminearum* onto flowering spikes in the greenhouse at the National Center for Agricultural Utilization Research, USDA, Peoria, IL. The DON-

producing isolate “GZ 3639” was provided by Dr. R. Bowden from Kansas State University. Mung bean liquid medium was used to produce conidial inoculum (Bai et al, 1999). The inoculated plants were enclosed in an inoculation chamber for 3 days on a greenhouse bench. The plants inside the chamber were misted daily with tap water by a hand sprayer. On the fourth day after inoculation, plants were returned to their original positions on the greenhouse benches. Greenhouse temperatures averaged 25 °C during the day with a range of 19 °C to 30 °C and 19 °C at night with a range of 17 °C to 21 °C. The greenhouse test was conducted in a completely randomized block design with two replications. Each replication had 6 plants. Disease symptoms were recorded as percentage scabbed spikelets at 6, 15, and 21 days after inoculation. Visual scab ratings were analyzed on a single plant basis. DON levels were evaluated for bulked seeds from the 6 inoculated plants of each entry in each replication.

Bulked seeds from inoculated plants were ground for DON analysis. Seeds from uninoculated spikes of selected lines were also analyzed as a control. The total ground sample was extracted by shaking with 5-mL acetonitrile/water (86:14, v/v) per gram of sample for 3 h. The extract was filtered into a vial using Whatman filter paper (Whatman International, Ltd, Maidstone, England). A fluorometric quantitation method (The Fluoroquant D Test Kit, Romer Laboratories, Inc., Union, Missouri) was used to analyze DON content in harvested grain. The detection limit for DON was 1 ug g^{-1}.

Amplified fragment length polymorphism (AFLP) markers were analyzed as described previously (Bai, et al, 1999). An AFLP linkage map was constructed with MapMaker software (Lander, et al, 1987) and QTL analysis was conducted with qGene software (Nelson, 1997).

Results and Discussion

Recombinant inbred lines differed in percentage of scabbed spikelets and incidence of seed infection (Table 1). Infection not only significantly reduced harvested seed number in all inoculated plants, but also the harvested seed weight. Seed weight reduction was low in Ning7840 and some resistant lines, but high in Clark and susceptible lines (Table 1).

DON was detected in all inoculated RILs, but not the control plants. A high level of DON accumulated in Clark, and a low level of DON accumulated in Ning 7840 (Table 1). In the RILs, the correlation between percentage of scabbed seed and DON content was high (r=0.72), indicating scabby seed is a good indicator for DON content. Only 4 RILs had a level of DON less than that in Ning 7840 (7 ug g-1), and about 50% of the lines had DON levels higher than in Clark (161 ug g-1, Fig 1). Transgressive segregation indicates several QTL may be involved in DON accumulation in wheat grain. Significant variation in DON levels between the two replications was also observed, indicating that replicated DON evaluation is necessary for reliable estimates of genetic effects.

A molecular map was constructed with 568 amplified fragment length polymorphism markers (Data not shown) and was used to map QTL for low DON. When DON data from the two replications were analyzed individually, one major QTL was identified in the same location in each replicate. This QTL explained about 23% and 26% of the total phenotypic variance,

respectively. When the average DON content over two replications was log-transformed and used for further QTL analysis, the QTL for low DON was mapped in the same region as in the separate analyses and explained about 25% of phenotypic variance (Fig 2). The major QTL for low DON was located in the same region as the major QTL for Type II scab resistance reported previously (Bai, et al, 1999). Since the major QTL for low DON explained only a small portion of phenotypic variance, some other QTL may also be involved in reduced DON accumulation. Spray inoculation with a high concentration of conidia provided much more inoculum and produced more severe scab than expected in natural conditions, therefore, high disease pressure in this study may have masked the expression of QTL with minor effects on DON levels in harvested grain. DON levels in infected grain from field experiments may need to be measured for further detection of other QTL.

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Fig. 1 Frequency distribution of DON contents in infected grain of F_{11} recombinant inbred lines from the cross of Ning 7840 / Clark

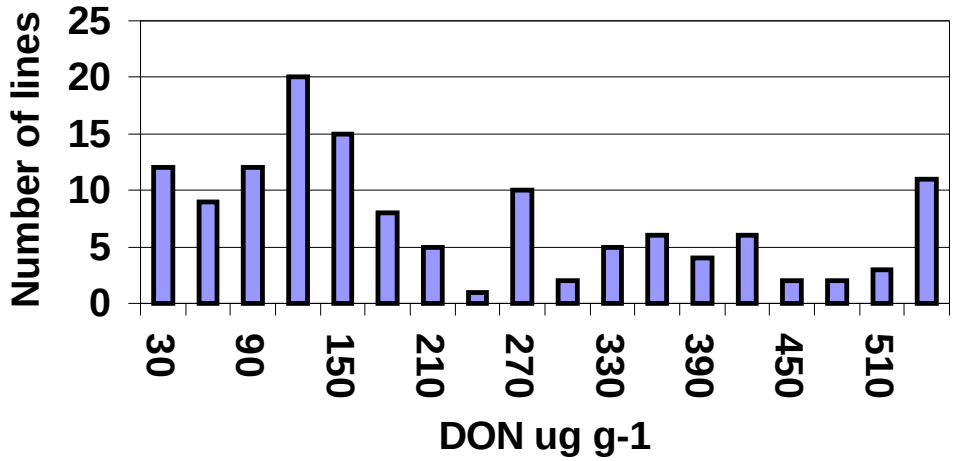
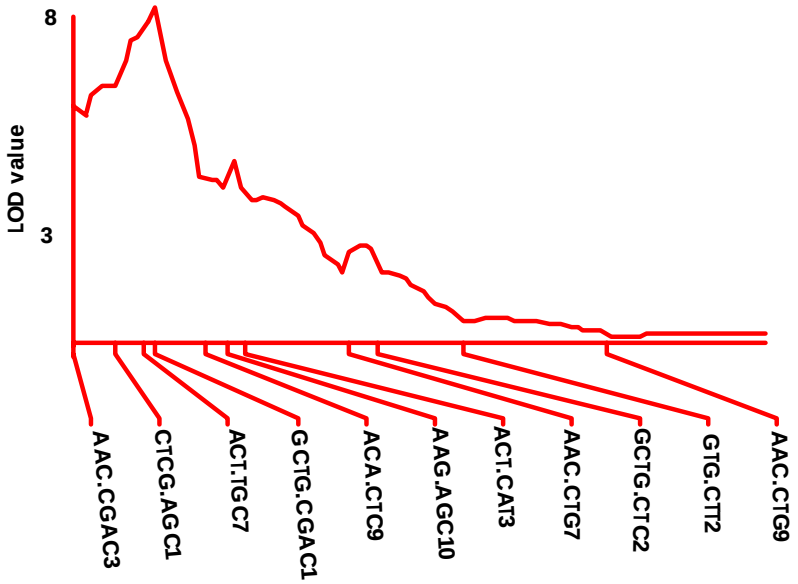


Fig. 2. LOD peak region of QTL for low DON on linkage group 7 as calculated by qGene software.



ESTABLISHMENT OF A USDA-ARS REGIONAL MOLECULAR GENOTYPING LABORATORY IN MANHATTAN, KS

GL Brown-Guedira*

USDA-ARS, Deptartment of Plant Pathology, Kansas State University, Manhattan, KS, 66506
*Corresponding author: PH: (785) 532-7260, E-mail: gbg@ksu.edu

ABSTRACT

The Agriculture Research Service of USDA has set the precedent in the last century of providing leadership and services when local resources have not been available to meet national needs in crop improvement. Regional ARS laboratories have been established to improve cereal quality and rust resistance, as well as the regional germplasm repositories that distribute and characterize germplasm collected from around the world.

Molecular genotyping promises to become the next critical tool in crop breeding and the development of new germplasm. New technologies will allow molecular marker-based selection to enhance plant breeding through improved accuracy and speed of genotype identification. Gains in efficiency and novel technologies have reduced the cost of a single observation by 5 fold. Molecular breeding tools represent the technology of promise as we enter the next era of plant breeding. As markets move away from a commodity basis toward a value-defined, end product basis, plant breeders must equip themselves with gene-specific markers which give them rapid access to traits of value.

To sustain and strengthen the USDA-ARS role in solving problems of regional and national scope it is critical to incorporate molecular genotyping technology. The adoption of this technology will position the USDA-ARS to facilitate the rapid deployment critically needed genes for wheat and barley.

A regional molecular genotyping laboratory for wheat that will serve public plant breeding programs in the US is being established. Currently, many genes for important traits are currently mapped due to efforts like the International *Triticae* Mapping Initiative. These markers are waiting full utilization in current breeding programs. The laboratory will be involved in:

■ Utilizing molecular markers linked to traits of value such as end use quality and resistance to insects and to diseases like *Fusarium* head scab, rust, wheat streak mosaic virus, and Karnal bunt in agronomically relevant populations in collaboration with public pant breeding programs. The laboratory will screen populations, individuals and lines provided by breeders; determine marker genotype then report back to breeders to facilitate speed and efficiency in germplasm release.

■ Creating marker profiles of cultivars and breeding lines that are used as parents and provide this information to the plant breeders so that markers can be selected that will be

useful in a particular cross. Marker profiles will also be cross-linked to other genetic information.

This laboratory is part of the Plant Science and Entomology Research Unit of the Grain Marketing Production Research Center in Manhattan KS which has lead scientists with expertise in germplasm development, mapping, and molecular biology. The unit already has an established wheat genomics facility that is a partnership with the Wheat Genetics Resource Center of Kansas State University. The major equipment necessary (automation and analysis) for a genotyping facility has already been purchased. Yearly funding of $250,000 has been obtained a scientist, support staff, and operating costs.

GENETIC ANALYSIS OF RESISTANCE TO *FUSARIUM* HEAD BLIGHT IN COMMON WHEAT

J. Chen[1]*, C.A. Griffey[1], M.A. Saghai Maroof [1], W. Zhao[1], W. Xie[2], T. Pridgen[1] and R.M. Biyashev[1]

[1]Virginia Polytechnic Institute and State University, Blacksburg, VA, 24061-0404;
[2]University of Minnesota, St. Paul, MN, 55108
*Corresponding author: PH: (540) 231-7624, E-mail: jianli@vt.edu

ABSTRACT

Fusarium head blight or scab is a devastating and economically important disease in all classes of wheat and barley worldwide. Insufficient or ambiguous knowledge concerning the amount of available genetic diversity and genetic mechanisms governing scab resistance, and lack of selectable molecular markers are constraints that greatly limit breeding efforts. Objectives of the current study are: 1) Determine the inheritance of scab resistance in three identified resistance sources; 2) Elucidate the genetic relationship between type II, III & IV resistance based on the segregation of scab severity, DON content and scabby seeds in four F_2 populations; and 3) Identify SSR molecular markers associated with type II, III and IV resistance in source W14 using F_2 population Pioneer 2684 x W14.

Three resistance sources, W14, Shaan 85 and Ernie identified in our previous studies (Griffey et al., 1998 & 1999), were crossed with susceptible soft red winter (SRW) wheat variety Madison and/or Pioneer 2684. The F_2 populations were evaluated in greenhouse tests using single floret inoculation procedures. W14 and Shaan 85 are improved type II resistance sources derived from Sumai 3 and may also possess other types of resistance. Ernie is a SRW wheat variety that lacks any of the known scab resistant sources in its ancestry (Chen et al., 2000a,b).

Two complementary genes with major effects were found to confer scab resistance in W14 and Shaan 85 based on similar segregation patterns of F_2 populations for type II, III and IV resistance characterized by disease severity, DON content and percentage of infected kernels (scabby seeds), respectively (Table 1 & 2). One to two genes were found to confer resistance in SRW wheat Ernie (Table 2). Segregation patterns observed in the four F_2 populations suggest that gene interaction is likely and may explain transgressive segregation as observed in this study and in those reported by other researchers (Buerstmayr et al., 1999 and 2000; Mesterhazy et al., 1999).

Significant positive correlations were found between disease severity (type II resistance), DON content (type III resistance), and scabby seeds (type IV resistance) based on segregation data from four F_2 populations. Highly resistant individuals with type II resistance were found to also possess type III and type IV resistance; however, about 25 % of individuals with type III and IV resistance didn't express type II resistance. Individuals with type IV resistance expressed type III resistance in most cases (Table 4).

The F_2 population Pioneer 2684 x W14 (150 individuals) was used to initiate mapping studies. Sixty-two SSR markers previously located on chromosomes 3B, 5A and 6B (Röder et al., 1998) were selected and evaluated for polymorphism between parental lines. Of the 62 SSR markers, 21 (34%) were polymorphic between resistant parent W14 and susceptible parent Pioneer 2684. Three markers, GMS389, GMS410, and GMS533, likely are associated with scab resistance and cumulatively accounted for 23, 18 and 19% of the phenotypic variance for type II, III and IV resistance, respectively in the current study (Table 3).

Correlation and regression analyses indicate that a specific association may exist between SSR markers and type of resistance (Table 3 & 4). GMS410 previously located on chromosome 5A (Röder et al., 1998) explained more of the phenotypic variance for DON production than for disease severity and scabby seeds; whereas, GMS533 explained more of the variation for disease severity and scabby seeds than for DON. GMS 389 explained more of the variation for disease severity and DON than for scabby seeds. GMS533 explained less of the phenotypic variance for resistance in the current study than in that of Anderson et al. (2001) and may be the result of multiple alleles being present at this resistance locus and/or variable linkage distances between the marker and QTL in different genetic backgrounds. This is supported by analyses of DNA polymorphism of the three markers evaluated in the current study among resistance sources Sumai 3, Funo (one of parents of Sumai 3), Shaan 85, W14, VR95B717, and SRW wheat varieties Ernie, Madison, Pioneer 2684.

Additional molecular markers, SSR and other types, will be evaluated in current and other mapping populations to identify putative QTLs associated with resistance, saturate chromosome regions associated with resistance, and develop a skeletal map. This research has a potential to identify new QTLs associated with scab resistance, provide additional markers linked to previously reported QTLs, and to identify markers that are effective across a variety of genetic backgrounds; all of which are essential for successful exploitation of marker-assisted selection.

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Table 1. Inheritance of scab resistance conferred by source W14 based on segregation of progeny for disease severity (%), scabby seeds (%) and DON content (ppm) in F_2 populations Madison x W14 and Pioneer 2684 x W14 assessed via floret inoculation in 1999 greenhouse tests.

	No. of Plants		Expected	χ^2	
Parents and cross	R *	S	Ratio	value	Probability
Madison x W14	64	74	7 : 9	0.2876	0.50 – 0.75
Pioneer 2684 x W14	80	126	7 : 9	1.8274	0.10 – 0.25
Madison x W14	62	76	7 : 9	0.0373	0.75 - 0.90
Pioneer 2684 x W14	115	91	9 : 7	0.0028	> 0.99
Madison x W14	83	55	9 : 7	0.6998	0.25 – 0.50
Pioneer 2684 x W14	86	120	7 : 9	0.2592	0.50 – 0.75

* R = Resistant and S = Susceptible progeny. Classification of resistant progeny was determined by the distribution and mean of the two parents, and based on the mean of resistant parents or the mean plus 0.5 to 1.5 times the standard deviation.

Table 2. Inheritance of scab resistance conferred by sources Shaan 85 and Ernie based on segregation of progeny for disease severity (%), scabby seeds (%) and DON content (ppm) in F_2 populations Pioneer 2684 x Shaan 85 and Pioneer 2684 x Ernie assessed via floret inoculation in 1999 greenhouse tests.

Parents and cross	R *	S	Expected Ratio	χ^2 value	Probability
Pioneer 2684 x Shaan 85	124	96	9 : 7	0.0012	> 0.99
Pioneer 2684 x Ernie	168	149	9 : 7	1.2342	0.25 – 0.50
Pioneer 2684 x Shaan 85	69	90	7 : 9	0.0001	> 0.99
Pioneer 2684 x Ernie	230	87	3 : 1	0.8843	0.25 – 0.50
Pioneer 2684 x Shaan 85	68	91	7 : 9	0.0289	0.75 – 0.90

*R = Resistant and S = Susceptible progeny. Classification of resistant progeny was determined by the distribution and mean of the two parents, and based on the mean of resistant parents or the mean plus 0.5 to 1.5 times the standard deviation.

Table 3. Multiple linear regression analysis for SSR markers associated with scab resistance in 150 F2 individuals from cross Pioneer 2684 x W14.

Markers	DF*	Probability			Coefficient of Determination			Favorable genotype**		
		Disease Severity (%)	Scabby Seeds (%)	DON Content (ppm)	Disease Severity (%)	Scabby Seeds (%)	DON Content (ppm)	Disease Severity (%)	Scabby Seeds (%)	DON Content (ppm)
GMS410	148	< 0.001	< 0.001	< 0.001	7.56	7.83	9.10	P	P	P
GMS389	148	< 0.001	< 0.001	< 0.001	10.53	7.35	9.50	W	W	W
GMS533a	148	< 0.001	< 0.002	0.039	9.92	6.40	3.94	W	W	W
GMS533b	148	< 0.001	0.002	0.009	10.51	6.60	4.57	W	W	W
All Makers	146	0.000	< 0.001	0.000	22.78	17.72	18.96	WP	WP	WP

* DF: Degree of freedom of error. ** W indicates W14 alleles that decrease disease severity, scabby seeds and DON content.P indicates alleles from Pioneer 2684.

Table 4. Linear correlation analysis between SSR maker data and disease data characterized by disease severity, scabby seeds and DON content in 150 F_2 individuals from population of Pioneer 2684 x W14.

				---- SSR Markers ----		
	Disease Severity	Scabby Seeds	DON Content	GMS389	GMS410	GMS533a
Scabby Seeds	0.5911**					
DON Content	0.5388**	0.8646**				
GMS389	- 0.3245**	- 0.2711**	- 0.3082**			
GMS410	0.2749**	0.2798**	0.3016**	-0.0567		
GMS533a	- 0.3150**	- 0.2529**	- 0.1984*	0.2756**	-0.055	
GMS533b	- 0.3241**	- 0.2568**	- 0.2137**	0.3077**	-0.0552	0.8089**

* Significant at P = 0.05; ** Significant at P = 0.01

EFFECTIVENESS OF MAS FOR SELECTION OF HEAD BLIGHT RESISTANCE IN SOFT RED WINTER WHEAT

J.M. Costa*, K. Salmon, A. Demianski, and K. Grant

University of Maryland, College Park, MD, 20742
*Corresponding author: PH: (301) 405-1317, E-mail: jc274@umail.umd.edu

ABSTRACT

The development of molecular genome analysis tools has created an interest in the use of marker assisted selection (MAS) in applied plant breeding programs. MAS may become an important aid to plant breeders in selection of superior genotypes. Instead of using phenotypic selection for a trait, once a tight linkage has been established between the trait and the marker, the marker can be used for selection. MAS may become especially useful for traits that are difficult to assay by phenotype such as scab or head blight resistance. In this research, we will examine the effectiveness of MAS for selection of germplasm with resistance to head blight of wheat by comparing field selection with MAS using Amplified Fragment Polymorphism markers (AFLPs) in two crosses (Ning7840/Pioneer 2643 and Ning 7840/Pioneer 2684). Bai et al. (1999) have recently identified AFLP markers linked to a major QTL controlling resistance to scab present in the cultivar Ning 7840. This QTL, located on chromosome 7 B, explained up to 60% of the variation in scab resistance. AFLP markers linked to this QTL will be used to indirectly select resistance genotypes in crosses between a resistant genotype and adapted SRWW genotypes. Breeding lines will be evaluated for agronomic traits and screened for the presence of the favorable AFLP alleles. Selection progress for scab resistance using MAS will be compared with phenotypic selection. We are currently screening the wheat parental lines for AFLP polymorphism.

EXPRESSION PATTERNS OF GENES FROM A HEAD SCAB INFECTED SPIKE CDNA LIBRARY.

John Fellers[1]*, Kristi Hill-Ambroz[1], Wanlong Li[2], and Bikram Gill[2].

[1]USDA-ARS and [2]Dept. of Plant Pathology, Kansas State University, Manhattan, KS, 66506
*Corresponding author: PH: (785) 532-2367, E-mail: jkn@alfalfa.ksu.edu

ABSTRACT

The sequencing of randomly selected cDNA clones has become a rapidly growing area for the identification of genes expressed in organisms with large genomes. A cDNA library was made from wheat spikes of the variety 'Sumai 3', 24 hr after inoculation with *Fusarium graminearum*. Isolated plasmids from 864 colonies were sequenced using automated DNA analysis. Quality sequence from 799 colonies was submitted for database alignment using BLASTX and alignment analysis revealed that the library contained 580 singletons. BLASTX alignments assigned putative function to 346 of the 580 singletons. The remaining singletons were aligned with genes of unknown function or had no significant alignment to sequences in the database. Of particular interest were 30 putatively assigned defense response genes, 7 disease resistance genes, and 7 stress response genes. Nylon arrays were made of the singletons and probed with cDNA made from mRNA of Sumai 3 spikes 0, 24, and 48 hr after infection. The expression patterns will be analyzed and reported.

FINE MAPPING OF A QUANTITATIVE TRAIT LOCUS FOR WHEAT SCAB RESISTANCE USING *PST*I-AFLP

[1]Guo, P-G, [2]Shaner, G.E, and [1]G-H Bai*

[1]Department of Plant and Soil Science, Oklahoma State University, Stillwater, OK 74074;
[2]Department of Botany and Plant Pathology, Purdue University, West Lafayette, IN 47907
*Corresponding author: PH: (405) 7449608, E- mail: bai@mail.pss.okstate.edu

ABSTRACT

In the previous study, we demonstrated that amplified fragment length polymorphism (AFLP) is a useful marker system for identification of wheat scab resistance QTL, and identified one major QTL for scab resistance from Ning 7840 by using *Eco*RI-AFLP. *Pst*I-AFLP markers ware reported to distribute more randomly in the corn genome than *Eco*RI-AFLP. Thus, we employed *Pst*I-AFLP markers to conduct further fine mapping in the major QTL region. The recombinant inbred lines (RILs) were derived from the cross between resistant cultivar Ning 7840 and susceptible cultivar Clark by single seed decent. The RILs were evaluated for scab resistance in the greenhouses by single spikelet inoculation. Wheat DNA from parents and 66 F_9 RILs was double-digested by *Pst*I and *Mse*I. Total 95 primer pairs were screened against the parents, and eighteen primer pairs showed a high level of polymorphism, therefore were used to analyze the RILs. Total 2,018 bands were amplified and 274 polymorphic bands were scored. Most of these markers were integrated into the existing *EcoR*I-AFLP map. The results indicated that *Pst*I-AFLP also randomly distributed in wheat genome. Addition of *Pst*I-AFLP markers not only increased genome coverage by filling the gaps in the *EcoR*I-AFLP map, but also increased the marker density. In the linkage group with major QTL, five *Pst*I-AFLP markers showed a significant association with scab resistance, and three of them explained high up to 70% of phenotypic variance in 66 RILs. Therefore, *Pst*I-AFLP is an effective tool for high-resolution mapping of scab resistance QTL in wheat.

FINDING QUANTITATIVE TRAIT LOCUS ASSOCIATED WITH FUSARIUM HEAD BLIGHT OF WHEAT USING SIMPLE SEQUENCE REPEAT MARKERS

[1]Anju Gupta, [2]P. E. Lipps*, and [3]K. G. Campbell

[1]Department of Horticuture and Crop Sciences and [2]Department of Plant Pathology, Ohio Agricultural Research and Development Center, The Ohio State University; Wooster, OH, 44691;
[3]USDA-ARS, Washington State University, Pullman, WA, 99164
*Corresponding author: PH: (330) 263-3843, E-mail: lipps.1@osu.edu

INTRODUCTION

Fusarium Head Blight (FHB) is a devastating disease of wheat (*Triticum aestivum*) reported since 1890. It is caused by fungus *F. graminearum* (teleomorph: *Gibberella zeae* Schwabe.). It has caused major grain losses all over the United States and worldwide. The loss is primarily due to floret sterility, poor to no seed filling, pink and shriveled tombstone kernels, low-test weight grains and presence of fungal mycotoxin-deoxynivalenol (DON) in the infected grains.

Molecular markers are powerful tools for marker assisted selection. Many markers closely linked with the disease resistance genes and quantitative trait loci (QTL) have been reported for many crops. QTL mapping will help locate genes that account for genetic variation in FHB resistance phenotypes. Restriction fragment length polymorphic (RFLP) and Amplified Fragment Length polymorphic (AFLP) markers have been used to identify FHB resistance QTLs in different genetic backgrounds. We have used Simple Sequence Repeat Markers to identify QTL associated with FHB resistance.

OBJECTIVE

Our main objective is to find quantitative trait locus associated with the FHB using two segregating populations, Ning7840 (resistant)/OH542 (susceptible) and Ning7840 (resistant)/Freedom (resistant). We are also interested in determining if the genes for resistance to FHB in Ning7840 and Freedom are similar or different.

MATERIALS AND METHODS

Two populations of $F_{2:3}$ lines developed by single seed descent method were used. These two populations were derived from a cross between Ning7840/OH542 (108 progenies) and Ning7840/Freedom (100 progenies).

Plant materials: Ten plants per family were evaluated for all the progenies of each population in the greenhouse. Seeds were sown in flats of soil in the greenhouse. Plants were vernalized for 60 days in a lighted cold room maintained day and night at 4^0C. Each germinated seed was transplanted individually. The greenhouse temperature varied from 19^0C to 30^0C during the day and 17^0C to 21^0C during the night.

The lines were also evaluated for resistance in the field at OARDC, Wooster, Ohio in 1999 for resistance to FHB. Lines were planted in a completely randomized block design with two replications each. Experimental units were 1m long and 30 cm apart (0.3 sq. feet). Patterson and Pioneer 2545 and OH542 were included as susceptible checks and Ning7840, Ernie and Freedom as resistant checks.

Inoculum Preparation: For greenhouse inoculation, fungal cultures from four aggressive *Fusarium graminearum* isolates were mixed in equal volume. The final concentration was adjusted to 10^5 conidia/ml. For field inoculations*, Fusarium graminearum* colonized corn kernels (Campbell and Lipps, 1998) were used as inoculum. The field was mist-irrigated daily throughout flowering.

I**noculation:** Hypodermic syringe inoculation technique was used for greenhouse inoculations. At anthesis, the center spikelet of each head was inoculated with a drop of freshly prepared conidial suspension (10^5 conidia/ml). Plants were maintained in a moist chamber at 100% relative humidity with temperatures ranging from 23^0C to 25^0C for three consecutive nights and then returned to the greenhouse bench.

Colonized corn kernels were spread in the field 18-21days prior to flowering. Heading dates were recorded as early, mid and late. 20 heads from each genotype were rated for %spikelet affected approximately 21 days after anthesis for severity ratings. Data was analyzed and compared with the greenhouse data.

Molecular Markers: DNA was isolated from parents and all 108 and 100 $F_{2:3}$ progenies of above two segregating populations. DNA extraction protocol perfomed was as described by Wang et al (1993). Primers for simple sequence repeat markers published by Roder et al (1996) were synthesized and screened for polymorphism among the three parents of above population. Priority was given to chromosomal regions that were found to be associated with QTL for FHB resistance in prior literature. Polymerase Chain Reaction (PCR) used was as described by Roder et al (1996) with a modification of 40 cycles of amplification instead of 45. Annealing temperature(T_M) was calculated for each primer individually based on the molecular weight. Amplified DNA products were visualized on a 4% superfine resolution agarose (SFR) run for 6-7hrs on the electrode. Ethidium bromide staining procedure was used. Primers showing polymorphism between three parents were used to evaluate all the progenies of the two populations.

Data: Inoculated heads were assessed for severity ratings as percentage of spikelet affected after 10 and 14 days in the greenhouse. FHB severity was recorded using a visual assessment scale (Stack et al, 1994, NDSU Extension) for greenhouse. Disease parameters recorded in the field were incidence, severity, visual kernel assessment scale (Stack et al, 1994, NDSU Extension), total kernel weight, percent scabby seed by weight and DON level (ppm).

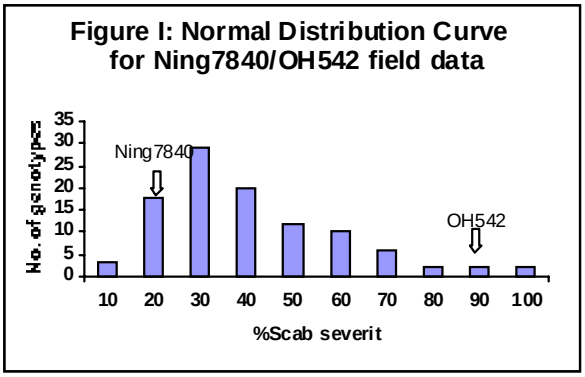
Statistical Model for Phenotypic Data Analysis: For both greenhouse and field, univariate plots for all the disease parameters were plotted independently using PROC UNIVARIATE in SAS(SAS institute version 6.03). One way analysis of variance (ANOVA) was conducted for the field and greenhouse data separately for the entire population using PROC GLM.

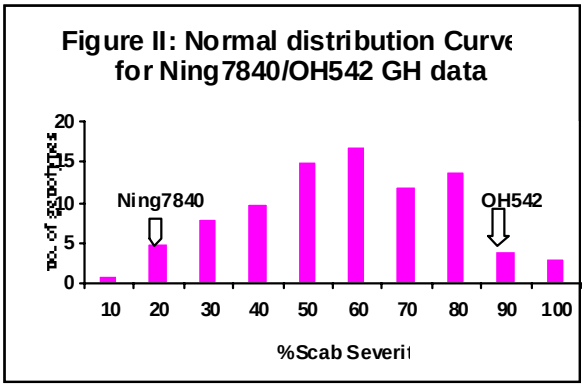
Pearson product movement correlations were calculated by PROC CORR to compare the disease severity ratings in different environments. The same procedure was also used to compare the disease severity ratings with other disease parameters from the field. In all cases, the correlation values were calculated from the means of genotypes at individual environment.

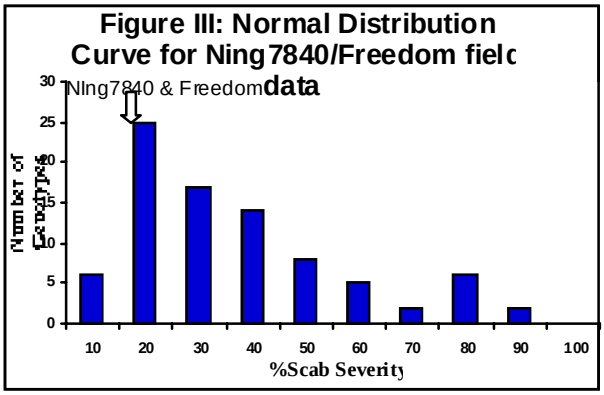
Statistical model for QTL Analysis: Quantitative trait anaysis was performed using SAS. One way ANOVA based on marker classes was conducted for each environment separately and on mean trait values over all environments using general linear model (PROC GLM) procedure for single codominant marker model for F_2 progeny. Rep and genotype within marker were considered random effect in the model. Error term was defined as gen(marker) to explain the uncontrolled variation.

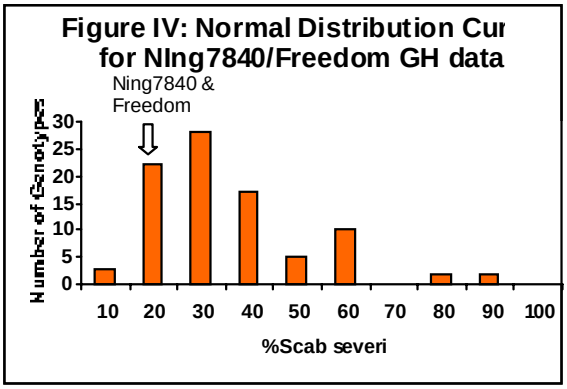
RESULTS

The tests for normality showed data for both the populations from greenhouse and field to be normally distributed. Data for other scab ratings from the field were also found to be normally distributed. Mean distribution curve for Ning7840/Freedom population and Ning7840/OH542 population for greenhouse and field are shown in figure I, II, III, IV. Field data of Ning7840/OH542 population is based on only one rep since 2nd rep was lost in the field due to mechanical failure of the planter. Greenhouse and field data were found to be moderately correlated for both the populations (r =0.37 & 0.35)









Genotypic data: Some of the SSR primers were found to be significantly associated with the trait in all the four experiments. Three other SSR primers located at 7BS and 2AS were found to be significantly associated with field FHB screening data(0.008 and 0.002 respectively) in the Ning7840/Freedom population. The test for significance was performed at 0.05 alpha level. Primers with low level of significance (0.10) are also reported in this preliminary report. All the progenies from above two populations are currently screened for a second year greenhouse testing for resistant to FHB. The final (2001) year data will help us in determining the true nature of QTL association. SSR markers significantly associated with one year greenhouse and one year field FHB disease screening are listed in Table I and II.

Table I: SSR Marker location and level of significance for association with FHB trait in Ning7840/Freedom population

SSR marker	Location	Significance level	
Xgwm493	3BS	0.05	*
Xgwm389	3BS	0.1	*
Xgwm285	3BS	0.1	**
Xgwm480	3AL	0.02	**
Xgwm296	2AS	0.003	*
Xgwm46	7BS	0.008	*
Xgwm518	6BS	0.1	***
Xgwm126	5AL	0.1	**
Xgwm186	5AL	0.1	*

Table II: SSR Marker location and level of significance for association with FHB trait Ning7840/OH542 population

SSR marker	Location	Significance level	
Xgwm389	3BS	0.05	**
Xgwm156	5AL	0.02	*
Xgwm2	3AS	0.01	*
Xgwm126	5AL	0.1	***
Xgwm533	3BS	0.03	**

* if the marker is significantly associated with the FHB screening data from field
** if the marker is significantly associated with the FHB screening data from greenhouse
*** if the marker is significantly associated with the FHB screening data from both greenhouse and field

A VISIBLE FUNGAL GROWTH APPROACH TO RAPID ANTIFUNGAL PROTEIN GENE PRETESTING

Hilburn, K.L.B., Baldridge, G.D., Bushnell, W.R., and Zeyen R.J.*

Department of Plant Pathology, University of Minnesota, St. Paul, MN, 55108
*Corresponding author: PH: (612) 625-4754, E-mail: richz@tc.umn.edu

OBJECTIVE

To develop a rapid pretest protocol for antifungal protein gene (AFP) constructs used in biolistic transformation of whole plants. The rapid system is based on a plant cell suspension culture approach to make it possible to test biolistic AFP constructs for their ability to stop or slow visible colony growth of the Fusarium Headblight fungus, *Fusarium graminearum*.

INTRODUCTION

Pre-testing of AFPs in suspension plant cell cultures is highly desirable because whole-plant transformation and headblight testing of adult transformants is expensive and time-consuming [Chen *et al.,* 1999; Smith *et al.*, 2000]. It often takes a minimum of eighteen months or more to test the efficacy of antifungal genes using whole plant transformation and adult plant screening procedures [Van de Mortel *et al.*, 1999]. In addition, individual AFPs are often not broad-spectrum and many isoforms may need to be tested. Plant suspension cell expression systems that accept a wide range of constructs are more desirable than other expression systems that require additional cloning steps. In addition some expression systems may not properly process biologically active eukaryotic antifungal proteins. Therefore a protocol using a plant suspension cell assay for pre-testing eukaryotic antifungal protein, biolistic constructs (AFPs) useful in genetic engineering of fungal disease resistant cereals [Bushnell *et al.,* 1998] is being developed. In this protocol plant suspension cell cultures transformed using biolistic constructs of antifungal genes are tested against the visible colony growth of the Fusarium Headblight fungus.

METHODS

The protocol uses Black Mexican Sweet Corn suspension cells (BMS) because they are easily transformed by microprojectile bombardment and are fast growing on MS solid and in liquid media [Murashige and Skoog, 1962]. BMS cells are ideal for protocol development because they respond to several commonly used plant promoters like the cauliflower mosaic virus promoter, maize ubiquitin promoter and others [Hilburn *et al.,* 2000]. In addition preliminary studies indicate that BMS cells can be stably transformed and subcultured for up to eight months without loss of transgene transcription capability (Table 1). Transgene viability during subculturing may depend upon the type of promoter used in a particular plasmid construct.

Plasmid and Promoter Driving GUS Expression And Cell Line Number	(~4 months after transformation)	(~8 months after transformation)	(~12 months after transformation)	(~17 months after transformation)
pKScBV GUS 13-3-1C	+	+	+	+
17-1-2A	+	+	+	+
17-1-2C	+	+	+	+
17-2-1C	+	+	+	+
19-1-2C	+	+	+	+
19-3-2C	+	+	+	+
pKUbi GUS 12-1-2D	+	+	+	+
13-1-1A	+	+	+	+
13-4-1B	+	+	+	+
16-4-1B	+	~+	~+	~+
18-2-2A	+	+	+	+
18-1-4E	+	+	+	+
19-3-2C	+	+	+	NS
pK35S GUS 12-1-2C	+	+	+	+
14-4-1A	+	+	NS	NS
13-4-1D	+	+	NS	NS
16-1-2C	+	+	NS	NS
19-2-1B	+	+	NS	NS

Table 1. Stability of GUS transformed BMS lines over time. BMS cells were co-transformed with a plasmid containing the GUS reporter gene and a plasmid containing the *nptII* gene as a selectable marker. Constructs with three differing promoters were used to drive the GUS gene. These were the Sugarcane Bacilliform Badna Virus promoter (ScBV), the maize ubiquitin promoter (Ubi), and the CaMV 35S promoter (35S). Stable lines were created and transferred weekly. Staining for GUS expression was used to determine GUS transgene stability throughout cell generations over time. A (+) designates positive GUS staining (blue product), a (~+) designates weak GUS staining, NS designates no sample available.

To develop this protocol four AFPs were chosen, all had previously documented antifungal activity in non-cereal plant systems [Datta *et al.*, 1999]. The AFPs were 1) rice chitinase [Zhu and Lamb, 1991], oat thaumatin-like protein [Lin *et al.*, 1996], and barley chitinase and barley glucanase [Leah *et al.*, 1991]. Several stably transformed BMS cell lines for each of four AFPs in constructs driven by the ScBV promoter [Tzafrir *et al.*, 1998] were created. To accomplish this, suspension cultured BMS cells were collected on cellulose filter pads, placed on solid MS media and transformed by microprojectile bombardment. The *nptII* gene (paromomycin resistance) was used as a selectable marker in co-transformation with all AFP constructs. After cell line selection, BMS-AFP lines were placed into MS liquid culture and grown in the presence of the paromomycin selector. A week before their use in the fungal growth assay procedure, BMS cell lines were transferred to liquid MS media without paromomycin [Hilburn *et al.*, 2000].

BMS-AFP suspension lines were verified for AFP mRNA transcription by RT-PCR. For preliminary study, a single BMS line, representing a single transformation event, was selected for each AFP. These BMS-AFP cultures were used to form cell "lawns" on cellulose filters. The AFP-BMS cell lawns were placed on solid MS media and centrally inoculated with a macroconidia suspension of known concentration from a virulent strain of *Fusarium graminearum.* The growth of the fungus (colony diameter) on each AFP-transformed cell line was recorded daily during an 8-day period.

PRELIMINARY RESULTS AND DISCUSSION

In replicated preliminary experiments none of the initial four AFPs, from single event BMS transformed cell lines, slowed or stopped the growth of the FHB fungus (Fig. 1). This occurred even though they transcribed mRNA for their AFP transgene. Although replicated, these results should be considered preliminary since there are more AFP-BMS cell lines from independent transformation events yet to be tested. Nevertheless the results are not unexpected since a closely related fungus, *F. solani* is well known for not responding to specific isoforms of chitinase, glucanase and other AFPs [Shewry *et al.*, 1997].

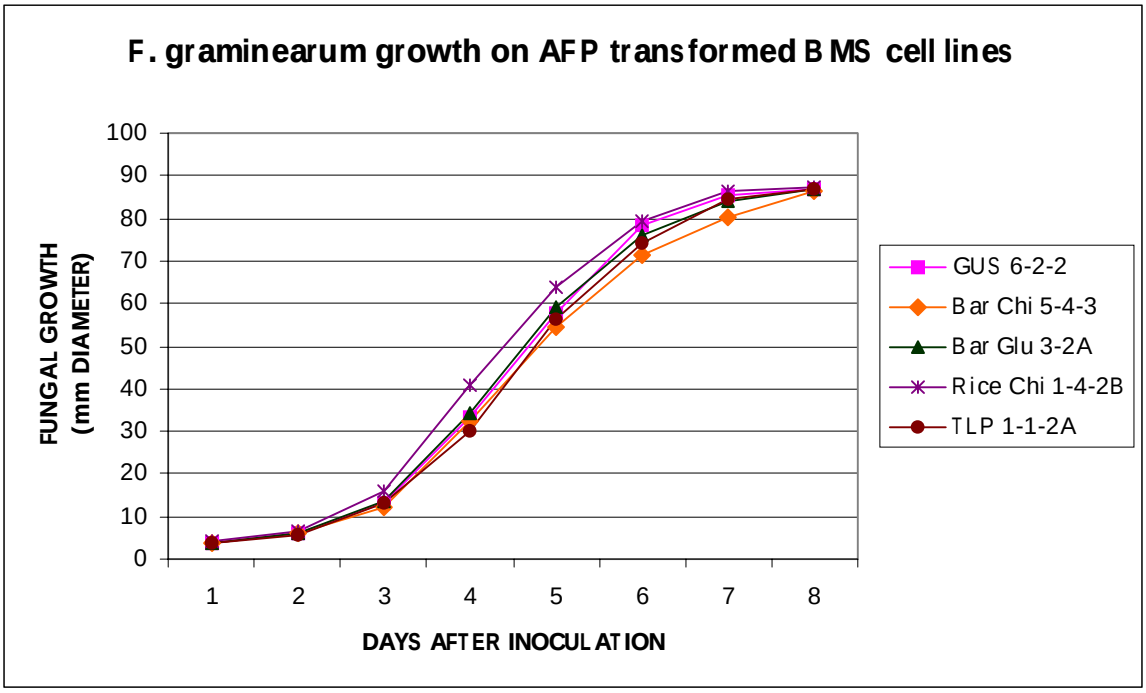


Figure 1. Growth of *F. graminearum* on AFP transformed BMS cell lines. Stable BMS suspension culture lines transcribing mRNA for their AFP were collected on filters and placed on solid MS media. These BMS cell lawns were then center inoculated with a macroconidia suspension of *F. graminearum*. Fungal growth measured as colony diameters were recorded daily, for 8 days.

A confounding factor is potential variability between AFP-BMS lines. Transformed BMS-AFP cell lines from single transformation events may differ in growth rates and in final transgene expression. Therefore more AFP-BMS lines from existing stocks and verified for transgene transcription by RT-PCR, (5 for each AFP) will be screened to verify preliminary results. In addition, *F. graminearum* grows well as saprophyte on MS media and may simply be over-growing and ignoring AFP-BMS cells by using the sucrose carbon base in the MS media as a substrate. Therefore, all future testing will be done on solid MS media minus sucrose. Preliminary experimentation revealed that BMS cells stay alive and active for up to 7 days on MS media without sucrose.

Also, it is well known that combinations of AFPs can act synergistically to halt fungi. The classic example is chitinase and b - 1,3 glucanase, which together can synergistically arrest growth of certain fungal species [Datta *et al*., 1999; Shewry *et al.*, 1997]. Therefore, mixtures of AFP transformed BMS lines will be made and tested as composite lawns against the growth of *F. graminearum.*

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GENETIC TRANSFORMATION OF BARLEY WITH GENES FOR SCAB RESISTANCE

M. Manoharan[1], T. M. Hohn[2], and and L. S. Dahleen[3]*

[1]Department of Plant Sciences, North Dakota State University, Fargo, ND, 58105; [2] Syngenta, Inc., Research Triangle Park, NC, 27709; [3]USDA-ARS Northern Crop Science Laboratory, Fargo, ND, 58105
*Corresponding author : PH: (701) 239-1384, E-mail: dahleenl@fargo.ars.usda.gov

ABSTRACT

Fusarium head blight (FHB), caused primarily by *Fusarium graminearum*, has been one of the most destructive diseases of barley since the early 1990s, resulting in huge economic losses for the growers. Of particular concern is the production of deoxynivalenol (DON), a pathogen virulence factor, which is harmful to humans and livestock. It has been proposed that increasing plant tolerance to DON could improve resistance to FHB while at the same time reducing DON accumulation in grain. *FsTRI* 101 (TriR), encodes a 3-OH trichothecene acetyltransferase that converts DON to a less toxic acetylated form while *PDR*5, an ATP-binding cassette transporter, acts as a efflux transporter, shunting DON across the plasma membrane from the interior of the cell. We have transformed the commercial malting barley cultivar Conlon with the *TRI* 101 and *PDR*5 genes with the aim of eliminating/reducing DON level in the infected grain. Ten day old callus derived from immature embryos was co-bombarded with the herbicide resistant gene, *bar*, as a selectable marker. After several rounds of selection on bialaphos medium, putative transgenic plants were regenerated. The transgenic nature of these plants were confimed by Southern analysis. Work is in progress to determine the expression of the introduced genes. Some of the regenerated plants were tetraploid. We are trying to convert transgenic tetraploid plants into diploid through conventional crossing.

IDENTIFICATION OF QTLS FOR SCAB RESISTANCE IN BARLEY

Mesfin, A.[1], G.J. Muehlbauer[1]*, D.C. Rasmusson[1], R. Dill-Macky[2], T. Walsh[1],
C.D. Gustus[1], and K.P. Smith[1]

[1]Department of Agronomy and Plant Genetics and [2]Department of Plant Pathology,
University of Minnesota, St. Paul, MN, 55108
*Corresponding author: PH: (612) 625-6228, E-mail: muehl003@tc.umn.edu

ABSTRACT

The current epidemic of *Fusarium* head blight (scab) in the northern Great Plains has significantly reduced the availability of malting barley grown in this region. Resistant cultivars are the most economical means to control this disease. DNA markers associated with scab resistance can be used to enhance early generation selection in a breeding program. The objective of this research is to identify RFLP and SSR markers that flank genomic regions associated with scab resistance. A population of 128 F_4-derived lines from the cross Fredrickson (moderately resistant)/Stander (susceptible) was evaluated for scab resistance. 148 RFLP and SSR markers were mapped in this population. Markers associated with a major QTL for scab resistance were identified on chromosome 2.

OPTIMIZING THE EXPRESSION OF CANDIDATE ANTI-*FUSARIUM* PROTEIN GENES IN HEXAPLOID WHEAT

P.A. Okubara[1], T.M. Hohn[3], R.M. Berka[4], N.A. Alexander[2], Z. Wang[5], L.P. Hart[5], and A.E. Blechl[1]*

[1]USDA-ARS, Western Regional Research Center, Albany, CA; [2]USDA-ARS, National Center for Agricultural Utilization Research, Peoria, IL; [3]Syngenta, Inc., Research, Inc., Research Triangle Park, NC; [4] Novo Nordisk Biotech, Inc., Davis, CA;
[5]Michigan State University, East Lansing, MI, 48824
*Corresponding author: PH: (510) 559-5716, E-mail: ablechl@pw.usda.gov

OBJECTIVES

The aims of this project are to generate lines of hexaploid wheat expressing genes that encode candidate antifungal (AF) proteins, and to optimize the activities of these proteins in tissues for effectiveness against Fusarium head blight (FHB). Our short-term objective is to increase the expression of our candidate genes in wheat floret organs.

INTRODUCTION

One approach to combating FHB is the introduction of genes encoding components of the host defense response pathway and proteins having antifungal activity. Using the biolistic method of genetic transformation, we have introduced six candidate AF protein gene constructs into the regenerable cultivar Bobwhite (Table I). Two of the genes encode proteins that are intended to reduce the toxic action of deoxynivalenol (DON), and four of the genes encode pathogenesis-related proteins that have the potential to disrupt the cellular architecture of the pathogen, *F. graminearum*. All of the genes are regulated by the constitutive maize *Polyubiquitin-1* promoter/first intron (UBI, Christensen and Quail 1996). This paper describes the assessment of steady state mRNA levels in transformed wheat lines containing each of the constructs. We summarize our findings on molecular features of the AF genes that might account for their generally low expression levels and our plans to improve fungal gene expression in wheat. The engineering of two novel candidate anti-*Fusarium* genes (Table II) for expression in monocots is also discussed.

Table I. Summary of candidate genes and their expression in transgenic wheat lines

A. Candidate AF genes.

Gene	Origin	Mode of Action	# Lines	Lines	Reference
FsTRI101	*F. sporotrichioides*	DON acetyltransferase	4	4	McCormick *et al*.
PDR5	*S. cerevisiae*	DON transporter	11	4/7 tested	Balzi *et al*.
tlp-1	*T. aestivum*	membrane disruption	4	3	Rebmann *et al*
FvGlu	*F. venenatum*	glucan degradation	6	3	unpublished
FvChi1	*F. venenatum*	chitin degradation	8	4	unpublished
FvChi2	*F. venenatum*	chitin degradation	3	2	unpublished

MATERIALS AND METHODS

Qualitative RT-PCR was carried out according to Altenbach (1998), using 200-600 ng total RNA from endosperm (15 to 25 days post anthesis) or glume (15 days post anthesis), and either the RNA PCR Core Kit (Perkin Elmer) or the OneStep RT-PCR Kit (Qiagen). For semi-quantitative RT-PCR, total RNA was diluted to 5-40 ng per reaction for amplification of *tlp* and actin transcripts, and 200-600 ng per reaction for amplification of all other transcripts. Primers for the amplification of wheat actin transcripts were CACTGGAATGGTCAAGGCTG (ActA) and CTCCATGTCATCCCAGTTG (ActB). The actin RT-PCR primers were derived from three *Triticum* expressed sequence tags (GenBank accession nos. BE398871, BE425854, BE492306) and are specific to monocot actin sequences (not expected to amplify fungal, insect or human actin sequences).

To enhance the expression of several *Fusarium* AF genes, we are modifying the three nucleotides immediately upstream of the ATG start codon (start codon context), the flanking upstream nucleotides (5' leader), and the frequencies of specific codons within the coding region. Modifications (Table II) to the candidate AF genes *FsTRI101*, *TRI12*, FvGlu, and DONPep2 are being carried out using synthetic primers and PCR (e.g., see Ho *et al*. 1989).

For the transient assay, embryogenic calli from wheat cv. Bobwhite or barley cv. Golden Promise were co-bombarded with UBI::*uid*A (GUS) and either UBI::*FsTRI101*, UBI::*tlp*, or UBI::FvEndo. After incubation for up to 48 hours, RNA was isolated and semi-quantitiative RT-PCR of the AF mRNAs, GUS, and actin transcripts was carried out as described above.

RESULTS AND DISCUSSION

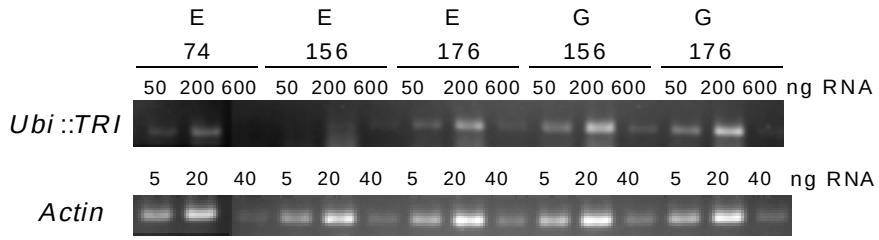
Thirty six independent wheat lines carrying one of the six antifungal gene constructs have been identified using the polymerase chain reaction (PCR) (Table I). Of these, a total of 20 transformed plants accumulated AF gene transcripts (mRNA) in endosperm, detectable by the reverse transcription-polymerase chain reaction (RT-PCR) method.

We observed plant-to-plant variations in mRNA levels for each of the candidate AF genes. In general, our RNA blot analyses (not shown) and RT-PCR experiments indicated that the expression of a *tlp* gene from wheat (Rebmann *et al*. 1992) was significantly higher than that of candidate genes derived from *Fusarium* or *Saccharomyces*, even though all of the genes

were regulated by the *Ubi* promoter/first intron. Although the levels of gene expression needed for effective action against *F. graminearum* have yet to be determined, high levels of expression are generally sought.

Semi-quantitative RT-PCR was used to distinguish among the different lines exhibiting transgene expression and to identify transformed lines expressing the highest steady-state levels of AF mRNAs (e.g., Fig. 1). Actin mRNA levels were monitored to normalize the amplification of the AF transcripts among different RNA samples. *In planta* endosperm expression levels ranged from 2- to 5-fold in independent lines expressing the same AF gene. *tlp-1* mRNA was about 10-fold more abundant than transcripts encoded by genes from *Fusarium* species. Where samples were available for testing, expression of the AF gene in glume tissues was very similar to that in endosperm. The glume is one of the first sites of *Fusarium* infection (Pritsch *et al*. 2000) under high inoculum pressure.

Fig. 1 RT-PCR detection of *TRI101* mRNA in endosperm (E) or glume (G) tissues harvested from three transgenic lines (74, 156, 175).



Our mRNA analyses indicated that the wheat *tlp-1* transcripts were significantly more abundant in wheat endosperm than were transcripts of the four *Fusarium* AF genes. To determine whether this was due to undesirable features within the fungal genes, we examined each for codon usage and consensus processing elements. The mRNAs deduced from the four fungal genes were free of known plant intron splice consensus elements, U-rich (e.g., Ko *et al.* 1998) or AU-rich segments (Haseloff *et al*. 1997; Iannacone *et al*. 1997) associated with monocot introns and transcriptional termination, and plant polyadenylation signals (Joshi 1987; Mogen *et al*. 1990). The GC content of the coding regions of the *Fusarium* AF genes ranged from 53% to 57 %, which compared favorably to the GC content of 39 non-redundant genes (containing over 11,300 codons) expressed in wheat leaves, wheat endosperm, and pollen of wheat, barley and maize. However, the *Fusarium* and monocot genes differed subtly in the usage of specific codons, and this might account for the lower accumulation AF gene transcripts of fungal origin in wheat tissues. Based on these analyses, we plan to change two of our constructs as shown in Table II.

Two new candidate AF genes, *TRI12* and DonPep2, are being modified for expression in wheat and other monocots. TRI12, a trichothecene efflux transporter from *F. sporotrichioides* (Alexander *et al*. 1999), is encoded by a 1.8 kb open reading frame. Owing to its smaller size and more favorable codon preference, the *TRI12* gene provides a promising alternative to *PDR5*. A novel peptide, DonPep2, is one of two DON antagonists that interfere with the toxic action of DON *in vitro* (Yuan *et al*. 1999). The peptide has been adjoined to the green fluo-

rescent protein for expression in plants. Modifications to *TRI12* and DonPep2 for expression in wheat are summarized in Table II.

Table II. Proposed gene modifications for enhancement of mRNA accumulation in monocots

Gene Name	Description of modification(s)
FsTRI101	start codon context from AAA to GCG with CA-rich 5' leader
FsTRI12	start codon context from AAG to GCG with CA-rich 5' leader
FvGlu	start codon context from ACT to ACC start codon context from ACT to GCC monocot codon usage
DONPep2	start codon context from ACA to GCG with CA-rich 5' leader and monocot codon usage

In order to test whether these proposed changes in 5' leader sequences or codon usage are effective in increasing the expression of fungal gene constructs in wheat, we are developing a transient expression assay. Such an assay will serve as a more rapid and tractable evaluation system than whole plant transformation. In preliminary experiments, wheat embryogenic calli were co-bombarded with UBI::*uid*A (GUS) plasmid in combination with either UBI::*tlp-1* or UBI::FvEndo plasmid. Transcripts of *uid*A, *tlp-1*, FvEndo, and endogeneous actin were readily detectable 48 hours after bombardment in our initial RT-PCR analyses, indicating that quality RNA from both introduced and endogenous genes can be isolated in suitable quantities. Barley embryogenic calli were co-bombarded with UBI::*uid*A (GUS) plasmid in combination with either UBI::*TRI101*, UBI::*tlp-1*, or UBI::FvEndo plasmid. Calli generated from embryos of barley cv. Golden Promise divide more rapidly than those from the wheat cv. Bobwhite and yield more material for bombardment. We are currently testing the suitability of barley callus tissue in transient expression assays. Assay conditions will be optimized using both wheat and barley tissues, and bombarded calli will be evaluated for reproducibility of expression. If relative expression levels of the AF genes in the transient assay correspond to those observed *in planta*, then new AF gene constructs can be evaluated within days rather than months.

ACKNOWLEDGMENTS

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GENOMICS EFFORTS TO UNDERSTAND FUSARIUM HEAD BLIGHT IN WHEAT

Thérèse Ouellet*, Hanhong Dan, Sharon Allard, Hélène Rocheleau, Tricia Glassco, Anju Koul, and Linda Harris

Eastern Cereal and Oilseed Research Centre, Agriculture and Agri-Food Canada, Ottawa, Ontario, K1A O06
*Corresponding author: PH: (613) 759-1658, E-mail: ouellettr@em.agr.ca

ABSTRACT

The main goal of the project is to identify and characterize genes from wheat which are involved in the host-pathogen interaction in fusarium head blight (FHB), to determine which gene pathways are modified in resistant sources and which pathways could be manipulated to play a key role in resistance. The wheat cultivar Frontana, a Type I resistance source from Brazil, has been used for the initial efforts. A suppressive subtraction hybridization (SSH) library has been made from Frontana heads inoculated at mid-anthesis with a *Fusarium graminearum* spore suspension and sampled 24 hr after inoculation. The subtraction was done with RNA from Frontana heads sprayed with water, to enrich for messages induced by the fungal infection. About 1800 ESTs have been sequenced and 1000 ESTs have been screened to select for genes with differential expression in inoculated heads. The differential expression pattern of potential candidate clones has been confirmed using Northern analysis.

We are also interested in the identification and characterization of genes from *Fusarium graminearum* which are involved in the host-pathogen interaction and in fungal pathogenicity. cDNA libraries have been constructed from *Fusarium graminearum* at various developmental stages and under different growth conditions, including mycelia growing on rich media, in Ahigh DON production@ liquid media, and on infected plant material, from asexual spore suspensions, and from developing fruiting bodies. Sequencing of ESTs from those libraries has been initiated.

PRELIMINARY CHARACTERIZATION OF WHEAT EVENTS HARBORING NOVEL TRANSGENES FOR SCAB RESISTANCE

S.J. Sato[1], J.A. Schimelfenig[2], S. Mitra[3], T.E. Clemente[1,3,4], A. Mitra[2], M. Dickman[2], J.E. Watkins[2], and P.S. Baenziger[3]*

[1]Center for Biotechnology; [2]Department of Plant Pathology; [3]Department of Agronomy; and
[4] Plant Science Initiative, University of Nebraska, Lincoln, NE, 68583
*Corresponding author: PH: (402) 472-1358, E-mail: agro104@unlnotes.unl.edu

ABSTRACT

The wheat biotechnology program at the University of Nebraska uses an *Agrobacterium*-mediated transformation protocol to deliver transgenes to wheat. The protocol is a modification of that reported by Cheng et al. (Plant Physiol. 115:971). Transformation frequencies range from 1% to 3% on a T_0 plant in soil per explant (immature embryo) basis. The advantages of an Agrobacterium-mediated protocol over a direct DNA delivery technique include lower copy number of the foreign DNA element, lack of integration of fragmented copies, and the lower probability of plasmid vector sequences outside the gene of interest region being integrated into the genome. We have successfully utilized the system to introduced foreign genes into three spring wheat genotypes, Bobwhite, Sakha 206 and UC703. Additional genotypes are currently being evaluated.

We have introduced into wheat a number of potential fungal resistance genes as a strategy to control *Fusarium* Head Blight. These include three antiapoptotic genes, IAP, ced-9 and Bcl-xl that may provide an avenue to modulate *Fusarium* pathogenesis and one direct antifungal peptide, lactoferrin. Data will be presented on inheritance of the transgenes in wheat, along with preliminary results on disease development from greenhouse inoculations on the derived transformants.

TARGETED EXPRESSION A THIONIN GENE TO INHIBIT GROWTH OF *FUSARIUM GRAMINEARUM* IN BARLEY

Ronald W. Skadsen[1]*, Puthigae Sathish[2], Jianming Fu[2], Maria Laura Federico[2], Heidi Kaeppler[2]

[1]USDA/ARS, Cereal Crops Research Unit, 501 N. Walnut St., Madison, WI, 53705;
[2]Agronomy Department, University of Wisconsin, Madison, WI, 53706
*Corresponding author: PH: (608) 262-3672, E-mail: rskadsen@facstaff.wisc.edu

OBJECTIVES

1) Produce *Fusarium*-resistant barley through genetic transformation with specifically targeted native antifungal protein genes. 2) Develop specific promoters for lemma/palea and pericarp tissues. 3) Develop expression vectors that combine these promoters with sequences to target hordothionin and other antifungal genes to the intracellular space. 4) Examine the tissue preference and routes of *Fusarium* infection.

INTRODUCTION

F. graminearum continues to destroy much of the U.S. barley and wheat crops. The production of mycotoxins (e.g., DON) by *Fusarium* makes the harvest unsuitable for food, feed or malting. Genetic transformation with antifungal protein genes may be a viable method for introducing biochemical resistance. Low concentrations (5 micrograms/ml) of hordothionin (HTH) completely suppress germination and growth of *F. graminearum* spores (Skadsen *et al.*, 1998). However, HTH is found only in the starchy endosperm. If HTH can be placed in external tissues such as the lemma/palea and pericarp, it may provide a barrier to *F.g.*

Whereas HTH is associated with protein bodies (Carbonero et al., 1980; Ponz et al., 1983), leaf thionin is associated with the vacuole and cell wall (Reimann-Phillip *et al.*, 1989). These can be induced by fungi and have antifungal properties (Florack and Stickema, 1994). The Arabidopsis *Thi2.1* gene is inducible by pathogens, and its over-expression leads to resistance to *F. oxysporum* (Epple *et al.*, 1997). *HTH* expressed in tobacco enhanced resistance to some pathogenic fungi but not others (Carmona *et al.*, 1993; Epple, *et al.*, 1997). However, the non-seed thionins apparently do not protect field barley from *F.g.* Thionin precursors (Ponz et al., 1983) contain an N-terminal signal peptide and a 64 amino acid C-terminal acidic protein (AP). Both are processed away, leaving a mature protein of 45 amino acids.

To effectively express anti-*Fusarium* genes, it is necessary to determine the route of *F.g.* infection. In a review of *Fusarium* (Parry *et al.*, 1995), the only histological study of *F.g.* (Pugh *et al.*, 1933!) noted that hyphae penetrate wheat kernels both inter- and intracellularly. *F. oxysporum* hyphae grow through the middle lamella and collapse adjoining cells, prior to penetration (Kroes *et al.*, 1998). Penetration of wheat stem cells by *F. culmorum* involves growth through the intracellular space (Ebrahim-Nesbat *et al.*, 1991). We have conducted studies with a strain of *Fusarium* (gfp/Fusarium) transformed with the green fluorescent protein gene of jellyfish (*gfp*; Skadsen *et al.*, 1999). Soon after spores become established on the lemma, hyphae grow rapidly to the tip of the floret. Hyphae then infect the anthers and

proliferate within the florets of young florets. In older florets, hyphae rapidly proliferate on the extruded ovary epithelial hairs; the anthers support comparatively little growth. Hyphae grow downward and cover the pericarp. Hyphae also penetrate the lemma and grow directly into the pericarp within 48 h from inoculation. It is therefore essential to express antifungal genes in these exposed tissues.

Constitutive expression is being used to test the effectiveness of antifungal genes. These results will be incorporated with ongoing research, which has produced a lemma-specific promoter and several other pericarp-specific genes. Once the constitutively expressed antifungal genes can be demonstrated to resist *Fusarium*, they will be coupled with tissue-specific promoters. This may eventually allow HTH and other antifungal proteins to be produced only in these tissues. Besides HTH transformants (see Results), we have also produced transformants carrying the gene for another antifungal permatin protein, BARPERM1 (Nuutila *et al*., 1998; Skadsen *et al*., 1999) and characterized the expression of this gene (Skadsen *et al*., 2000). Our transformants have not produced BARPERM1 protein in vegetative tissues. We have located a probable cause for this (below) and will use this information to improve HTH and permatin transformation.

MATERIALS AND METHODS

Tissue-specific promoter cloning, vector construction, transformation and expression

The differential display technique was used to detect genes expressed in the lemma/palea or pericarp but not in flag leaves. Products were used to probe blots of lemma/palea, pericarp and leaf RNAs. Tissue-specific gene candidates were cloned and sequenced, and the corresponding nuclear genes were purified from a Morex genomic BAC library (Andy Kleinhofs, Washington State U.). Deduced promoter regions were subcloned and ligated to a *gfp* reporter gene (Jen Sheen, Harvard). Transient expression studies of promoter activity were conducted with the pAHC17 vector, which contains the *Ubi* promoter and first intron but no selectable marker (Christenson and Quail, 1996). We inserted *gfp* behind the *Ubi* promoter. Vectors for stable constitutive expression of *gfp* and antifungal genes were prepared from the Ubi/GUS+Ubi/BAR vector pAHC25 (Christenson and Quail, 1996) by replacement of the GUS gene. Vectors were attached to gold particles and used to transform barley through the biolistic (gene gun) approach (Wan and Lemaux, 1994), with media modifications described by Dahleen (1995). Screening of putative transformants was conducted using PCR on leaf DNA extracted by the CTAB procedure. Particle bombardments of lemmas, pericarps and leaves are being conducted with candidate tissue-specific promoters, linked upstream from *gfp*. Later, promoter/*HTH* fusions will be constructed. Stable transformants will be tested for *Fusarium* resistance. In the past, we have transformed the Golden Promise cv. However, this does not lend itself to adequate field testing because its spikes do not fully emerge from the boot. We will now transform the two-row Conlon cultivar, which has been shown to transform and regenerate (Dahleen, 2000).

HTH antibodies and Subcellular targeting of HTH

HTH-1 was inserted into a pET vector in order to produce the full-length HTH protein. This failed to produce protein in a pET/*E. coli* system. Because the *HTH-1* sequence has two

nearby start codons and a segment of 5' UTR, it was hypothesized that these may inhibit expression. *HTH-2* was produced by removing the sequence upstream of the second start codon. This produced high levels of HTH fusion protein (fused with pET thioredoxin). The fusion protein was purified and used to produce antibodies in rabbits. If effective (see Results) these will be used to confirm expression at the protein level. Our initial thionin expression vector utilized the *HTH-1* cDNA clone. All constructs were inserted into pAHC25 behind the *Ubi* promoter, after removing the GUS sequence. The 3' UTR of the *HTH-1* sequence was removed and joined with the NOS termination sequence. Because of our success with *HTH-2* in the pET system, we inserted it into the pAHC25 vector. *HTH* sequences were detected by PCR. RT-PCR was used to determine whether HTH genes were transcribed in transformants.

In order to understand the requirements for targeting HTH to the intracellular space and/or to the secretion pathway, three constructs were inserted into pAHC25, behind the HTH signal sequence. Construct 1 diverts targeting from the vacuole by inserting a synthesized KDEL ER retention signal (reviewed in Gomord and Faye, 1996) between the N-terminal signal peptide (SP) and GUS. Construct 2 contains the SP and mature protein followed by GUS. Without the AP, the HTH-GUS fusion may be directed to the intracellular space. Construct 3 contains the SP alone, followed only by the GUS sequence. The vectors were bombarded into etiolated coleoptiles. After 48 h, coleoptiles were stained for GUS activity or analyzed for secreted GUS activity (MUG assay), relative to intracellular MDH.

Route of* Fusarium *invasion

A strain of *F.g.* transformed with *gfp* was produced by Tom Hohn (Novartis, NC (formerly at USDA, Peoria, IL)). The hyphae display a green fluorescence under short-wave blue light (Skadsen *et al.*, 1998; Bushnell *et al.,* 1999). We explored several methods for visualizing the initial penetration events. Paraformaldehyde fixation and cryostat sectioning were found to be ineffective. Lemmas are infected for 6 h and then peeled into fine tissue strips, preserving conidiospore attachments. These were viewed by confocal microscopy (Keck Neural Imaging Center, Univ. of Wisconsin), but excessive "flare" prevented viewing at the epidermis. We are now experimenting with the use of tissue strips set into enclosed deep well slide chambers.

RESULTS AND DISCUSSION

A previously unknown gene, *D5*, has been cloned and found to be expressed only in the lemma/palea and embryo (Sathish et al.,1999). The promoter was used to drive the expression of a *gfp* reporter gene in a *D5*/*gfp* construct. In transient assays, expression was found in lemmas and not in leaves (Sathish, et al., 2000). As an internal control, the Ubi/GUS vector was simultaneously bombarded. GUS was expressed well in both leaves and lemmas. Several other lemma/palea- and pericarp-specific genes have been cloned. We have successfully transformed Golden Promise with *HTH-1* and have so far found five transformants that produce *HTH-1* mRNA in seedling leaves, on northern blots. However, in RT-PCR analyses, only one produced appreciable levels of *HTH-1* mRNA. We have recently bombarded embryos with *HTH-2* and have several regenerating transformants.

Subcellular targeting constructs were tested by transient expression assays. Although weak, all secreted GUS activity (MUG assay) into the apples, whereas no MDH activity was secreted (cell leakage control). The unaltered GUS control did not secrete, and had only localized GUS staining spots. In all of the non-control bombardments, the entire coleoptile turned blue after GUS staining. We are now trying to determine whether artifacts could have caused these results.

HTH antibodies were tested on western blots of developing seed proteins and purified HTH protein. They did not cross-react with purified (45 amino acid) HTH mature protein, although they did react with two seed proteins of the proper size to represent the unprocessed HTH and the AP. Two new constructs will be made to obtain a sequence that will react with mature HTH.

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CONSTRUCTION OF GENOMIC LIBRARIES ENRICHED WITH MICROSATELLITE SEQUENCES

Q. J. Song, E. W. Fickus, and P. B. Cregan*

Beltsville Agricultural Research Center, USDA-ARS, Beltsville, MD, 20705
*Corresponding author: PH: (301)5047208, E-mail: creganp@ba.ars.usda.gov

ABSTRACT

In bread wheat, Simple Sequence Repeats (SSRs) or microsatellite markers provide a higher incidence of polymorphism than other marker types such as RFLP and RAPD. SSR markers are usually developed by the following process: genomic DNA is digested with different restriction enzymes; size selected fragments are (400-700 basepairs) are ligated into a vector and used to transform *E. coli*, the clones are transferred to membranes and screened with different SSR-containing probes; clones that putatively contain an SSR are then sequenced and PCR primers are designed to the flanking regions of the SSR sequence. The construction and screening of this type of library has two notable limitations: firstly, the screening is time consuming, secondly, because of the low frequency of SSR containing clones very large numbers of clones must be screened. To overcome these problems, an elaborate method for [CT/GA]n enrichment of wheat has been used based on the marker selection method which carried out as follows:

Genomic DNA of Chinese Spring was digested with restriction enzymes *Sma*I, *BsrB*I, *Nla*IV, *Cac8*I, and *ECI136*II, and size selected on an agarose gel. DNA fragments in the 400 to 750bp range were isolated from the gel. Purified DNA fragments were ligated into the *SmaI* site of pBluescript. Ligations were propagated in bacterial strain JMG1 which combined mutations at the *dut* and *ung* loci. The presence of these two mutations permits the incorporation of uracil in place of thymidine in DNA replicated in the strain. The phagemid library was infected with VCSM13 helper phage, this permits the recovery of this library as single stranded (ss) DNA with uracil frequently substituted for thymidine. Primer extension was carried out by using the ssDNA as a template and the 5' phosphorylated $[CT]_{15}$ oligo as a primer. DNA synthesis, preceded by an optional short annealing step, is performed using *Taq* DNA polymerase. The products of this primer extension reaction were treated with T4 DNA ligase and then transformed into an *E. coli* strain XL2-Blue maintaining wild type genes at the *dut* and *ung* loci. Under these circumstances, the uracil substituted ssDNA will be restricted from growing by the host encoded uracil-N-glycosylase (the product of the *ung* locus), while the primer extended products are capable of replicating. Libraries were plated at a density of 100-200 clones per Petri plate. Random clones from both primary and marker-selected libraries were picked and cultured in a 96 well plate with LB liquid medium, after transferring to the membranes, the clones were hybridized with a $[CT]_{15}$ probe labelled with ^{32}P.

A total of 366 [CT]n containing clones were observed out of 2592 marker-selected clones. Compared with the reported frequency of 0.1% to 0.2% for [CT]n, the library enrichment for [CT]n is about 70 to 140 fold. Eighty-three positive clones were randomly selected and

sequenced. The mean repeat length of [CT]n is 14. The clones with prefect repeats =10 were 64% of the total. After being assembled on the AutoAssembler, only two clones were found to have identical sequences, the remaining clones were unique. Further screening for other motifs is in progress.

DEVELOPMENT AND PHYSICAL MAPPING OF MICROSATELLITE MARKERS IN WHEAT

Sukhwinder-Singh[1], Wanglong Li[1], Qijian Song[3], P. Cregan[3],
G. L. Brown-Guedira[2] and B. S. Gill[1]*

[1]Department Plant Pathology and [2]ARS-USDA, Department Plant Pathology,
Kansas State University, Manhattan, KS 66506;
[3]ARS-USDA, BARC-West, Beltsville, MD, 20705
*Corresponding author: PH: (785)532-1391, E-mail: bsg@ksu.edu

ABSTRACT

Physical mapping of BARC and BARCM primers:

Microsatellite markers, also referred as simple sequence repeat (SSR) markers, are very efficient marker system for molecular breeding. We are broadening wheat genetic marker system by identification and physical mapping of new microsatellites.

We have determined the chromosome location of 141 microsatellite markers amplified by 70 BARC and 71 BARCM primer pairs designed in the laboratory of Dr. Perry Cregan (USDA/ ARS, Beltsvilles, MD) using nulli-tetrasomic lines of Chinese Spring. Polymorphism was screened among five wheat cultivars or lines, such as CS, Opata85, Synthetic wheat W-7984 (parents of ITMI mapping population) WL711 and HD29 (parents of Karnal bunt mapping population). All BARC primer pairs were polymorphic between Opata85, and synthetic wheat W-7984, whereas all BARCM primer pairs produced monomorphism. Fifteen percent (11/70) of BARC and three percent (2/71) BARCM primers detected polymorphism between wheat lines WL711 and HD29. It was possible to assign 75 loci with 57 BARC and BARCM primer sets on wheat chromosomes (Table 1). A maximum number of loci (7) was mapped on chromosomes 5B and 7D. Six loci were placed on each of chromosomes 6B, 6D, and 7A. No markers mapped on chromosomes 1D and 4B. Sixty-eight percent of BARC and 20 percent BARCM primers were mapped on individual wheat chromosomes.

Development of new microsatellite markers using the EST database:

Rapid growth of wheat EST database provides A new resource for development of new microsatellite markers. We searched the database at Microsoft interface for di-and tri-nucleotide repeat motifs {(AC)n, (AG)n, (AT)n, (CG)n, (CT)n, (GT)n, (AAC)n, (AAG)n, (AAT)n, (ACC)n,(ACG)n,(ACT)n, (AGC)n,(AGG)n,(AGT)n,(ATT)n,(CCG)n,(CCT)n,(CGG)n,(CGT)n, (CTG)n,(CTT)n,(GGT)n, and(GTT)n}. One hundred and ten putative microsatellites were identified from 15,000 ESTs. One hundred pairs of primers were designed manually.

Out of 100 primers tested on wheat lines HD29 and WL711, 74 percent amplified a PCR product and six percent were polymorphic between these two lines. WL711 and HD29 are highly susceptible and resistant wheats to Karnal bunt pathogen respectively. Twenty-two of these primer sets were tested on nulli-tetrasomic lines of CS and twelve were mapped to individual wheat chromosomes.

Wheat EST database is expected to grow to 100,000 or more. We anticipate designing and testing a total 600 primer sets. Based on preliminary results, we expect 70 percent of the primers will amplify PCR products for grand total of 420. Based on our preliminary physical mapping data, at least 50 percent of these new markers should map as distinct loci on individual wheat chromosomes. Therefore, we expect to develop 200 new microsatellite markers from the EST database. Identifying microsatellite markers from the EST database is less time consuming and more cost effective than other methods of isolating microsatellite markers.

All the microsatellite markers developed in our laboratory along with those developed from the lab. of Dr. P. Cregan will be physically mapped using CS nullitetra lines, di-telosomic and deletion lines.

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Table 1. Physical mapping of BARC/BARCM microsatellite markers on wheat chromosomes using Chinese Spring nulli-tetrasomic set.

Chromosome*	A	B	D
1	BARC48, BARC83 BARC17, BARCM48	BARC61, BARC80 BARC88	-
2	BARC08, BARC50 BARC51, BARC48	BARC55, BARC13 BARC16, BARC18, BARCM64	BARC54, BARC08 BARC28
3	BARC57, BARC19, BARC12, BARC45	BARC77, BARC68, BARC73, BARC84, BARCM44	BARC77, BARC42, BARC71
4	BARC78, BARC52	-	BARC69
5	BARC40, RARCM32	BARC59, BARC69 BARC04, BARC16 BARC58, BARCM18, BARCM32	BARC44
6	BARC90	BARC24, BARCM26 BARCM06, BARC67 BARC67, BARCM06, BARCM67, BARCM31, BARCM68	BARC05, BARC54, BARC25, BARCM30, BARC21, BARC62
7	BARC70, BARC49 BARC29, BARCM25 BARCM04, BARCM34	BARC32,BARC90 BARC29,BARCM26 BARCM67	BARC09,BARC70 BARC26,BARC06 BARC52,BARC76 BARCM24

* Note some SSR amplified products on more than one chromosome. For example BARC48 amplified products on chromosome 1A and 2A. BARC08 on chromosomes of 1A and 1D

DEVELOPMENT OF STSS AND SNPS LINKED TO FUSARIUM HEAD BLIGHT RESISTANCE OF WHEAT USING AFLPS AND ANTIFUNGAL GENE ANALOGS

[1]I. Vroh Bi*, [1]F. L. Kolb, [1]L. K. Boze, [2]G. Bai, and [3]L. L. Domier

[1]University of Illinois, Urbana, IL, 61801; [2]Oklahoma State University, Stillwater, OK, 74078; and [3]USDA-ARS, Urbana, IL, 61801
*Corresponding author: PH: (217) 244-2568, E-mail: vroh@uiuc.edu

OBJECTIVES

To design user-friendly PCR markers for scab resistance genes, using AFLP markers and sequences from genes encoding antifungal thaumatin-like proteins.

INTRODUCTION

Breeding for scab resistance currently relies mainly on phenotypic assessments of infection symptoms. Given the inherent difficulties in field-based screening, user-friendly molecular markers associated with scab resistance genes would facilitate selection. Polymerase chain reaction (PCR) based markers such as sequence-tagged sites (STSs) and single nucleotide polymorphisms (SNPs) have been applied in a number of analyses (Inoue et al. 1994; Wang et al. 1998). These allele-specific PCR markers are usually designed from previously mapped low-copy DNA markers, or built directly from sequences of known genes. Sequences of disease resistance genes of several plant species share structural motifs. These conserved domains can be used to isolate similar resistance genes from a given genome with degenerate primers (Shen et al. 1998). In previous work, AFLP markers linked to three QTL for scab resistance were identified (Bai et al. 1999). The aim of the present study was: (1) to convert the AFLP markers into STSs and SNPs, and (2) to design additional allele-specific PCR markers for scab resistance based on sequences of genes having similarity to antifungal thaumatin-like protein genes (Vigers et al. 1992; Chen et al. 1999).

MATERIALS AND METHODS

The mapping population consisted of 133 $F_{8:11}$ recombinant inbred lines (RILs) derived from 'Ning 7840'/Clark. 'Ning 7840' has strong type II resistance and Clark is very susceptible to scab spread within the spike. Disease screening methods, phenotypic data, and AFLP protocol have been described (Bai et al. 1999). AFLP fragments were recovered from polyacrylamide gels, amplified, gel purified, cloned, and sequenced. All the fragments were hybridized by Southern blotting on parental genomic DNA digested by *Eco*RI, *Hind*III, and *Hae*III to select low-copy sequences. Antifungal genes were isolated from 'Ning 7840' and Clark using degenerate primers based on amino acid motifs conserved among several thaumatin-like protein genes. All the degenerate primers contained deoxyinosine at the redundant third position of codons, and full nucleotide redundancy was used at the 3' termini. SNPs were designed on point mutations within sequences. The STSs and SNPs were integrated into an established AFLP map (Bai et al. 1999) using Mapmaker 3.0 (Lander et al.

1987) for linkage mapping, and Qgene 3.04 (Nelson, 1997) for interval analysis and regression.

RESULTS AND DISCUSSION

A total of 9 AFLP fragments linked to scab resistance were isolated from polyacrylamide gels. These fragments included 7 markers located around a major QTL, and 2 markers linked to two minor QTL (one marker per QTL). Together the three QTL explained up to 60% of the genotypic variation of scab resistance (Bai et al. 1999). Southern blotting revealed that 6 out of 9 fragments were low-copy AFLP clones. As expected, the sequence of each clone was flanked by the corresponding *EcoR*I and *Mse*I primer sequences. Based on the sequences of the AFLP markers, two types of allele-specific primers were designed. The first type was internal to the AFLP selective primers. In the second type, at least one of the direct and reverse primers included the AFLP selective primer sites. The internal primers did not give any allele-specific amplification. Instead, primers designed on the original restriction sites identified five STSs reproducing the original polymorphisms. A sixth STS amplified a fragment of the expected size in both 'Ning 7840' and Clark. These fragments revealed a single base change at position 91 (a thymine in Ning 7840 replaced a cytosine in Clark). The point mutation was used to design a SNP designated SNPmaj for its location in the major QTL region. Interval analysis showed that two allele-specific markers STSmaj and SNPmaj mapped to linkage group LG1 spanning 81.2 cM, with maximum LOD score value of 21.84 (Figure 1A). STSmaj and SNPmaj were designed from the sequences of the AFLP markers GCTG/CGAC1 and AAC/CGAC3, respectively. These AFLPs were linked to a major QTL for type II resistance (Bai et al. 1999). The allele-specific markers mapped 4.8 cM and 2.9 cM away from their respective AFLP markers (Figure 1B). Another allele-specific marker, STSmin derived from the AFLP marker CTCG/CTC4, mapped in the linkage group LG2 covering 73.6 cM (data not shown).

A number of thaumatin-like protein genes were isolated from 'Ning 7840' and Clark. Alignment of sequences identified 15 point mutations (insertions and substitutions). The allele-specific marker SNPtha, linked to the minor QTL in linkage group LG2 was derived from a 633 bp insert containing a segment of a thaumatin-like protein. The R^2 and P values of the four allele-specific PCR markers for area under disease progression curve (AUDPC) and percentage of scabbed spikelets (PSS) at 21 days after infection in F_{10} RILs are given in Table 1. STSmaj and SNPmaj located in the major QTL region explained individually 35 to 36% of the phenotypic variation of scab infection. The effects of allele substitution at STSmaj and SNPmaj are significant (0.01 to 0.001 level probability) on both AUDPC and PPS in four generations (F_5, F_6, F_7, and F_{10}). The minor QTL marked by STSmin and SNPtha on linkage group LG2 has marginal effects on scab resistance (Table 1), with LOD score value less than 3.0.

The markers STSmaj and SNPmaj can be individually multiplexed with STSmin in 25 PCR cycles. Reagent concentrations are the same for all markers, but SNPtha has a different annealing temperature. These four PCR markers should prove useful in selection for scab resistance. They will be validated in different crosses. Several other antifungal genes are being analyzed for mapping in the major QTL region.

Table 1. R^2 and P values of area under disease progression curve (AUDPC) and percentage of scabbed spikelets (PSS) at 21 days after infection for F_{10} generation of RILs.

Marker	Marker source	Allele source	AUDPC		PSS	
			R^2	P	R^2	P
STSmaj	AFLP(GCTG/CGAC1)	Clark	35	0	36.6	0
SNPmaj	AFLP(AAC/CGAC3)	Ning	35.1	0	35.2	0
STSmin	AFLP(CTCG/CTC4)	Ning	2.1	0.0996	2.6	0.019
SNPtha	Thaumatin-like protein	Clark	4.6	0.0135	4.2	0.063

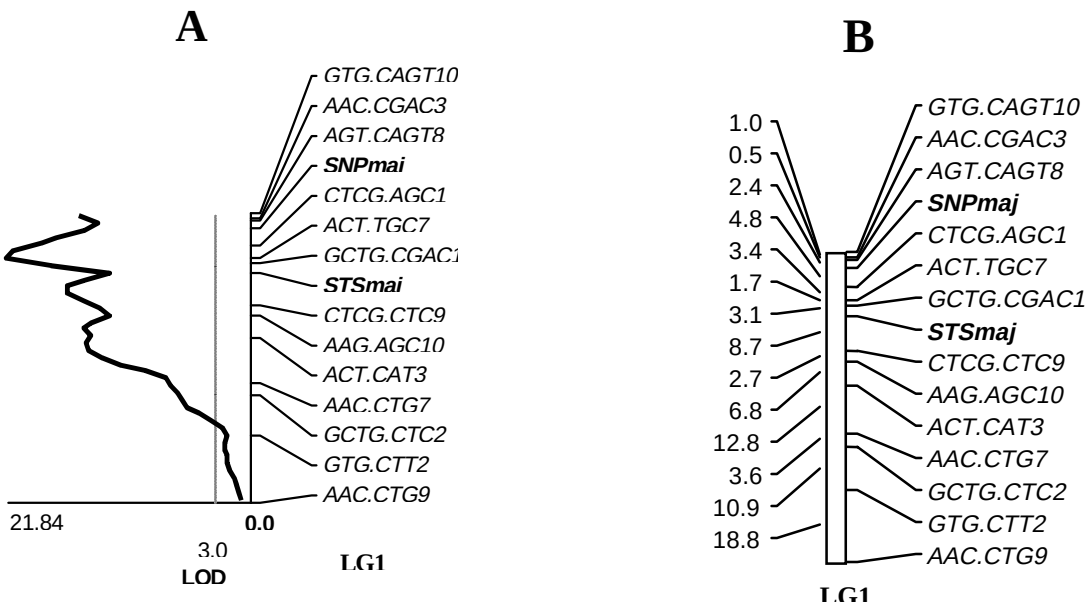

Figure 1. Interval analysis (A) and map of linkage group1 (B) carrying a major QTL for scab resistance in 'Ning 7840'/Clark recombinant inbred lines. The allele-specific markers SNPmaj and STSmaj are in bold.

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MICROSATELLITE MARKER DEVELOPMENT AND CONSTRUCTION OF A MICROSATELLITE ALLELE SIZE DATABASE OF ELITE AND SCAB RESISTANT WHEAT GENOTYPES: MEIOTIC MAPPING AT MSU AND RATIONALE FOR THE OVERALL PROJECT

*[1]R. Ward, [1]Chen Xin-min, [1]Shi Jian-rong, [2]Qijian Song, and P. Cregan[2]

[1]Department of Crop and Soil Sciences, Michigan State University, East Lansing, MI.; 48824-1325;
[2]USDA-ARS, Beltsville Agricultural Research Center, MD, 20705
*Corresponding author: PH: (517) 285-9725, E-mail: wardri@msu.edu

ABSTRACT

The unsuccessful search for potent scab resistance in elite U.S. wheat germplasm dictates that any new, potent resistance genes will be found in unadapted parents. Likewise, the limited number of lines amenable to transgenosis dictates that useful transgenes will also be initially available to breeders only in unadapted transgenic lines. Conventional breeding approaches for introgressing genes from unadapted parents generally requires several 'cycles' of backcrossing or modified backcrossing to dilute the frequency of undesirable donor alleles. The result is a large and costly gap between the resistance levels available to scientists, and those actually in use by growers.

Marker assisted selection is an effective means of rapid and directed elimination of unwanted donor alleles, or 'background selection' (Bernacchi et al., 1998a; Bernacchi et al., 1998b; Xiao et al., 1998). For this application, a marker system must exhibit high levels of polymorphism at loci evenly distributed throughout the genome. Co-dominant inheritance and multi-allelism are also desirable since heterozygotes are not masked and polymorphism is maximized. Finally, genetic analysis should require small amounts of DNA and must be amenable to automation to permit high-throughput genotyping. Microsatellite or simple sequence repeat (SSR) markers meet these requirements. SSR polymorphism is based on differences in the number of simple sequence repeats at loci defined by locus-specific PCR primers flanking the SSR sequence (Weber and May, 1989). Evidence in wheat indicates that SSR loci are abundant and well distributed, and the primer sequences for approximately 455 SSRs are now available including about 315 from the literature (Bryan et al., 1997; Donini et al., 1998; Korzun et al., 1999; Roder et al., 1998a; Roder et al., 1998b and others) and 141 from this project. This number of loci is inadequate to provide the genome coverage needed to optimize QTL discovery in wheat which has an estimated genome length of 3403 centiMorgans (cM) (calculated from the ITMI map on GrainGenes). This is true because it is unlikely that more than one-third of these 455 loci would be polymorphic in a given bi-parental cross. Thus, the average distance between loci would be 3403 cM / 152 loci = 22.4 cM between loci. Because marker loci are unlikely to be evenly distributed in the genome, many gaps of much greater than 22.4 cM would be anticipated. In soybean, with a genome length of about 2600 cM, a recent Genomics Whitepaper (http://129.186.26.94/Genomics/Soybean_Genomics.html) indicated the need to develop and map 1000 SSR loci in addition to the 1000 already available.

For full functionality, SSR loci should be annotated with three types of information: 1) primer sequences, 2) map position at several levels of resolution, and 3) the allelic status of relevant hexaploid and durum wheat accessions (i.e., primary sources of resistance, elite adapted breeding parents, and selected progeny). The last two types information are required for practical implementation to integrate markers into practical programs of QTL discovery and manipulation and background selection. The marker genotype data for selected progeny become part of the overall annotation database. These data help breeders avoid unnecessary marker assays with subsequent generations of background selection.

The USWBSI is funding a large scale effort to increase the number of SSR loci available in wheat and to initiate the mapping and allele databases. This poster describes the meiotic mapping underway at Michigan State University and our vision of how this combined project can acclerate deployment of scab resistance genes.

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GENETIC ENGINEERING WHEAT FOR SCAB RESISTANCE

Wyckoff, M.[1], L. Smith[1], G. Baldridge[2], R. Zeyen[2] and G.J. Muehlbauer[1]*

[1]Department of Agronomy and Plant Genetics; and [2]Department of Plant Pathology, University of Minnesota, St. Paul, MN, 55108

*Corresponding author: PH: (612) 625-6228, E-mail: muehl003@tc.umn.edu

ABSTRACT

Fusarium head blight (FHB) of wheat caused by *Fusarium graminearum* has resulted in serious economic losses due to reductions in yield and quality. Increasing the expression of antifungal protein (AFP) genes in wheat via genetic engineering is a promising approach for enhancing FHB resistance as well as resistance to other fungal diseases. Our objectives were to develop germplasm sources of transgenic wheat overexpressing the following AFP genes: wheat a-thionin, barley ribosome inactivating protein (RIP), barley PR-5 (thaumatin-like protein), and barley class-II ß-1,3-glucanase. Embryogenic calli of the spring wheat cultivar 'Bobwhite' were cotransformed via particle bombardment with one of the AFP genes and the plasmid pAHC25. PAHC25 carries the *bar* gene for selection on the herbicide bialophos and the reporter gene *uidA* for visual scoring of ß-glucoronidase (GUS) activity. For each AFP gene, between 700 and 1400 immature embryos were bombarded. Calli were selected and plants were regenerated on 5 mg/l bialophos for a minimum of 12 weeks. Between 80-300 plants were regenerated for each AFP gene. RT-PCR was performed on RNA isolated from T_0 and T_1 leaves and plants were found to express the wheat a-thionin, barley RIP, PR-5 and ß-1,3-glucanase AFP transgenes. Preliminary FHB disease severity data will be reported for a-thionin, barley RIP and ß-1,3-glucanase.

IDENTIFICATION, CLONING AND SEQUENCING OF ESTS RELATED TO FHB RESISTANCE OF WHEAT

D. H. Xing[1], Y. Yen[1]*, J. C. Rudd[2], Y. Jin[2]

[1]Department of Bio/Microbiology; and [2]Department of Plant Science, South Dakota State University, Brookings, SD, 57007
*Corresponding author: PH: (605) 688-4567, E-mail: yang_yen@sdstate.edu

ABSTRACT

One of big obstacles in fighting FHB epidemics is that little is known about the nature of FHB resistance, particularly at molecular level. Our research aims at addressing this problem and getting insight into the molecular mechanism of *F. graminearum*-wheat interaction. Our current objectives were to identify, clone, sequence and analyze ESTs related to FHB resistance by comparing the EST profiles of resistant and susceptible cultivars. Sumai 3 (resistant) and Wheaton (susceptible) were employed in this research. When the first anther spring out, a single middle floret of the spikes was inoculated with 20 ml of Fg4 isolate (70, 000 spores/ml) or water (as control). The treated plants were immediately transferred into mist chambers and cultured for 24 hours (for the treatments more than 24 hours). The inoculated spikelet and the other four spikelets immediately next to it were sampled 0, 2, 4, 8, 16, 32 and 64 hours after inoculation. The total RNA extraction was conducted using Tri reagent (Molecular Research Center Inc., Cincinnati, Ohio) according to the procedure provided by the manufacture. The RNAimage kit (GenHunter Corporation, Nashville, TN) was used for obtaining the EST profiles. The whole procedure provided with the kit was followed except that we used designed primers instead of random primers. The primers were designed according to the highly conserved “LRR” domain in the products of all known cloned resistance genes (the only exception is Pto gene). A total of 144 primer combinations were tested. Several gene expression patterns were observed: 1) constitutively expressed in Sumai 3; 2) constitutively expressed in Wheaton; 3) induced expression in *F.graminerum*-inoculated Sumai 3 and Wheaton only; 4) induced expression in *F. graminerum*-inoculated Wheaton only; and 5) induced expression in *F. graminerum*-inoculated Sumai 3 only. ESTs of the last category are most likely related to FHB resistant genes. Three such ESTs,

EST12G, *EST15AU* and *EST15AD*, were cloned with PCR-Trap cloning kit (GenHunter Corporation, Nashville, TN) and sequenced using ABI automatic sequencer. A sequence similarity-search of GeneBank data base revealed that *EST15AU* is 94% similar to part of a wheat mRNA for polypeptide elogation factor 1 beta'; *EST15AD* has three homologous regions (with 86% identity) with an EST sequence from a pathogen induced sorghum bi-color cDNA; *EST12G* is almost identical (with 99% identity) to a part of minus strand of a wheat gene for chloroplast ATP synthase CF-O subunit I and III. Confirmation of the accurate relationship of these three ESTs with FHB resistance by genetic analysis is on the way.

A MICROASSAY APPROACH TO RAPID ANTIFUNGAL PROTEIN GENE PRETESTING

R. J. Zeyen*, G.D. Baldridge, W.R. Bushnell, K.L.B. Hilburn

Department of Plant Pathology, University of Minnesota, St. Paul, MN, 55108
*Corresponding author: PH: (612) 625-4754, E-mail: richz@tc.umn.edu

OBJECTIVE

To develop a fast (less than 3-day) pretest protocol for antifungal protein gene constructs used in biolistic transformation of whole plants.

INTRODUCTION

A microassay protocol is under development for rapidly testing eukaryotic antifungal protein (AFPs) expression constructs of potential value in genetic engineering of cereals for resistance to Fusarium headblight. An approach using plant cell suspension culture and green fluorescent protein transformed *Fusarium graminearum* was selected. This made it possible to test quickly biolistic AFP constructs for their ability to stop hyphae of *F. graminearum* from attacking AFP-transformed cell clusters.

This rapid protocol can help select promising AFP candidates from the numerous AFPs available [Datta *et al.*, 1999; Shewry and Lucas 1997]. By pretesting AFPs it may be possible to speed development of Fusarium headblight resistant wheat and barley. Currently it is very expensive and time-consuming to screen AFPs using whole-plant transformation and adult plant disease testing [Bushnell *et al.* 1998; Chen *et al.,* 1999; Smith *et al.,* 2000; Van de Mortel *et al.,* 1999]. This microassay protocol offers the advantage of eukaryotic cells in which biolistic constructs can be used. It also means that we avoid extra cloning needed for bacterial expression systems, which themselves may not properly process eukaryotic proteins. With experience the microassay protocol can be used to test large numbers of potential AFP genes in commonly used biolistic constructs.

METHODS

The protocol is based on use of a hand-held biolistic gene gun and Black Mexican Sweet Corn suspension culture cells (BMS). These were transferred to cellulose filters and placed on MS solid media [Murashige and Skoog, 1962] in Petri plates, for ease of manipulation. BMS cells were co-transformed using microprojectile bombardment. A visual marker gene set construct for upregulating anthocyanin, and one or more AFP expression constructs were used in each experiment. A Helios (BIORAD) biolistic gene gun at 140 - 160 lbs./sq. in He_2 pressure delivered the plasmid constucts to the BMS cells. One-micron diameter gold particles coated with plasmid DNA (4 ug DNA/mg gold, 1.0 ug DNA per shot) was used as the gene carrier, other variables were as described in the BIORAD Helios handgun protocol.

After 24 hours, the AFP-anthocyanin co-transformed cells were inoculated with a *F. graminearum* isolate previously transformed with an *Aequorea victoria* green fluorescent

protein (GFP) reporter gene construct by Thomas Hohn - USDA, Peoria. Inoculation was done with macroconidia placed centrally on the BMS cell filter. Thirty hours later, BMS cell filters were examined microscopically for co-transformed cells (seen as brown or red cells or cell clumps) and for GFP fungal hyphae. Interaction sites were viewed with epifluorscent UV and near-blue illumination using an Aus Jena light microscope with appropriate filters and a dichroic mirror that allowed wavelengths above 510 nm to be seen. At the edge of small fungal colonies, where individual hyphae were distinguishable, transformed BMS cells were scored for fungal hyphal contact or fungal hyphal avoidance. Singly transformed BMS cell filters (anthocyanin upregulated only) were inoculated and data from were used as the control to which all other data was compared.

AFP plasmids tested contained the Sugar cane Badna Virus (ScBV) or maize ubiquitin promoters followed by a maize alcohol dehydrogenase intron or a maize ubiquitin 1 intron, the particular AFP coding sequence and the *Agrobacterium* NOS terminator. The antifungal protein genes tested were:

- pBScBV Rchit- rice chitinase transgene [Zhu and Lamb, 1991]
- pBScBV TLP1- oat thaumatin like protein cDNA [Lin *et al.*, 1996]
- pBScBV Barchit- barley chitinase cDNA [Leah *et al.*, 1991]
- pBScBV Barglu- barley glucanase cDNA [Leah *et al.*, 1991]
- pBScBv ArabPR5- Arabidopsis PR5 cDNA [Uknes *et al*., 1992]
- pUBK Tri101-Trichothecene 3-0-acetyltransferase cDNA [Kimura *et al*.,1998]
- pAHC WIR 2- wheat thaumatin-like protein cDNA [Rebman *et al*., 1991}

The anthocyanin visual marker (pPHI687) plasmid (Bowen, 1992) contained pairs of tandem Cauliflower Mosaic Virus 35S promoters and the maize alcohol dehydrogenase intron I to drive mRNA expression of transcription factors C and R which up-regulate expression of enzymes in the BMS cell anthocyanin pathway [Grotewold *et al.,* 1994].

To check for AFP transcription, primers were made for each AFP and RT PCR was done on extracts from BMS cells, removed from individual filters 30 h after transformation.

RESULTS AND DISCUSSION

RT PCR results revealed that AFP mRNAs were transcribed in BMS cells transformed with single and/or multiple expression vectors. From previous work we estimated an 80% or better co-transformation rate for our AFP constructs and the anthocyanin marker construct.

Five cellulose filters, containing BMS cells, were used for each gene construct in each individual experiment. Experiments with single or multiple AFP containing biolistic constructs were repeated a minimum of two or three times. BMS cell filters singly transformed

with the construct that upregulated anthocyanin were used as controls. Data from each experiment, with its appropriate anthocyanin control, were pooled and statistically analyzed and shown in Table 1.

Table 1. Pooled data from experiments with single or multiple AFP-containing biolistic constructs. All experiments were repeated a minimum of two or three times. Data from each experiment were pooled and analyzed. * = A significant different from Anthocyanin control at P<0.05, using student's t test.

Antifungal Proteins Tested	Number of Fungal Encounter Sites	Percent Hyphal Contact
Anthocyanin Control	**867**	**70**
Arabidopsis PR5	694	55*
Tri 101	739	50*
Wheat WIR 2	877	50*
Anthocyanin Control	**280**	**60**
Barley Chitinase	368	57
Barley Glucanase	380	51
Barley Chit/Gluc	315	55
Anthocyanin Control	**631**	**59**
Oat Tlp1	553	51
Anthocyanin Control	**458**	**55**
Rice Chit/Tlp1 Barley Chit/Gluc	593	50
Anthocyanin Control	**469**	**61**
Rice Chitinase	425	61
Rice Chit/Tlp1	445	62

Results from this microassay protocol show that *F. graminearum* contact was repelled by the antifungal proteins Arabidopsis PR5, Fusarium Tri 101 and wheat WIR 2. However the barley chitinase, barley glucanase, rice chitinase and oat Tlp1 AFPs were ineffective in repelling *F. graminearum* contact. Also, combinations of chitinase/glucanase or chitinase/Tlp 1 were ineffective.

The BMS cell transient assay of AFP efficacy has potential as a rapid means of evaluating AFPs for later use in whole plant transformation. It also could be used with other non-biotrophic fungal pathogens of wheat and barley to test the efficacy of various AFP candidates. At present we have no strong positive control AFP, like that used by Mitra *et al.* [2000] with which to compare candidate AFPs and to optimize our microassay protocol.

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CREATION OF AN AFLP MAP FOR IDENTIFICATION OF SCAB RESISTANCE GENES FROM WHEAT CULTIVAR WANGSHUIBAI

Xu Zhang[1], Peiguo Guo[2], Weizhong Lu[1], and Guihua Bai[2]*

[1]Institute of Agrobiological Genetics and Physiology, Jiangsu Academy of Agricultural Sciences, Nanjing, CHINA, 210014; [2]Department of Plant and Soil Sciences, Oklahoma State University, OK, 74078
*Corresponding author: PH: (405) 744-9608, E-mail: bai@mail.pss.okstate.edu

ABSTRACT

Wheat scab, mainly caused by *Fusarium graminearum,* is an important disease of wheat worldwide. Resistance genes from Sumai 3 and its derivatives Ning 7840 have been well characterized through molecular mapping approach. However, resistance genes in Wangshuibai are still a puzzle to be solved. To characterize the scab resistance genes in Wangshuibai, an amplified fragment length polymorphism (AFLP) linkage map was constructed with 104 F7 recombinant inbred lines (RILs) derived from the cross between resistant cultivar Wangshuibai and susceptible cultivar Alondra. For AFLP, DNA was digested with EcoRI and MseI restriction enzyme and corresponding primers were used for AFLP analysis. EcoR I primers were labeled with ^{33}P-?ATP and PCR-products were separated in a 5% polyacrymide gel. Total 207 AFLP primer pairs were screened for two parents, and 167 of them (80.7%) amplified scorable DNA fragments. In an average, each primer pair amplified about 8 polymorphic fragments between parents, indicating a relatively high level of polymorphism between the parents. A total of 32 informative primer pairs amplified about 410 bands segregating in the F7 RILs. About 250 markers have been mapped in 23 linkage groups covering a genetic distance of 2430 cM. The map will be further saturated with *Pst*I-AFLP and SSR markers and used to locate the QTL for scab resistance in cultivar Wangshuibai. This is first effort to map scab resistance genes from Wangshuibai, another important sources of scab resistance from China.

SSR MAPPING AND SUB-ARM PHYSICAL LOCATION OF A MAJOR SCAB RESISTANCE QTL IN WHEAT

W. C. Zhou[1], F. L. Kolb[1]*, G. H. Bai[2], G. Shaner[3], and L. L. Domier[4]

[1]Department of Crop Science, University of Illinois, IL, 61801; [2]Department of Plant & Soil Sciences, Oklahoma State University, Stillwater, OK, 74078-6028; [3]Department of Botany and Plant Pathology, Purdue University, West Lafayette, IN, 47907-1155; and
[4]Department of Crop Sciences, USDA-ARS-MWA, Urbana, IL, 61801
*Corresponding author: PH: (217) 333-9485, E-mail: f-kolb@uiuc.edu

OBJECTIVES

To identify SSR markers for a major scab resistance QTL mapped by AFLP markers in the previous study (Bai et al., 1999), and to locate the QTL to a specific sub-arm region of the chromosome.

INTRODUCTION

Molecular marker technologies provide an accurate approach to manipulation of quantitative traits such as scab resistance. Random amplified polymorphic DNAs (RAPDs), restriction fragment length polymorphisms (RFLPs), and amplified fragment length polymorphisms (AFLPs) have been used to map scab resistance genes (Bai, 1995; Bai et al., 1999; Waldron et al., 1999). Using RAPD markers with F_5-derived recombinant inbred lines (RIL), developed by single seed descent from Ning 7840 (resistant) and Clark (susceptible), Bai found two QTL controlling scab resistance on two linkage groups. The two QTL explained 18% and 6.1% of the total phenotypic variations respectively (Bai 1995). Four years later, Bai et al. (1999) screened 133 F_9 RILs derived from the same population with AFLP markers. One major QTL for scab resistance was identified, which explained almost 60% of the genetic variation for Type II scab resistance (Bai et al., 1999); however, the chromosomal location of this QTL was not determined.

MATERIALS AND METHODS

Plant Materials: Chinese Spring and derived aneuploid lines were used for physical mapping of SSR markers associated with scab resistance QTL. Thirty-five nullitetrasomic lines (missing lines were Nulli (N) 2A Tetra (T) 2B, N2AT2D, N4BT4D, N4BT4A, N4DT4A, N5BT5A, and N6BT6D); thirty-two ditelosomic lines (missing 2AL, 4AS, 5AS, 2BS, 4BL, 5BS, 3DS, 3DL, 5DS,and 7DS); and eight deletion lines for chromosome 3BS were kindly provided by Dr. John Raupp at the Wheat Genetics Resource Center, Kansas State University.

The mapping population was a set of 133 $F_{8:11}$ RILs from the cross of Ning 7840 with Clark developed by Bai et al. (1999). The phenotypic data for scab resistance and inoculation method were described in detail by Bai et al. (1999). DNA isolation was performed with the modified SDS method.

SSR analysis

SSR primers were synthesized by Life Technologies Inc. according to sequence information published by R‘der et al. (1998).

Mapping and data analysis

The computer program MAPMAKER (Lander et al., 1987), V3.0 for the MacIntosh, was used to calculate linkage distances and integrate SSR and AFLP marker data. Algorithms used a Kosambi mapping function (Kosambi, 1944) with a LOD of 3.0. Interval analysis was performed by using Qgene software (Nelson, 1997).

Physical mapping of SSR markers: Nullitetrasomic and ditelosomic lines were used to determine the chromosome and arm location of polymorphic bands by scoring the presence or absence of specific amplified SSRs. Deletion lines related to 3BS were used to further assign the sub-arm location of mapped SSR markers after the major QTL was located on chromosome 3B.

RESULTS AND DISCUSSION

Screening and mapping of polymorphic SSR markers between Ning 7840 and Clark:

n total, ninety-three SSRs were mapped, and thirty-four were polymorphic between Ning 7840 and Clark. When the mapping data were analyzed together with the AFLP mapping data from the same mapping population, we found that Xgwm 389, a marker on 3BS, was linked to the AFLP markers tightly associated with the major QTL. All other SSRs on 3B were then analyzed. Two SSRs on 3BS, Xgwm533, and Xgwm493, and one SSR, Xgwm340, on 3BL were polymorphic between Ning 7840 and Clark. Xgwm389, Xgwm533, and Xgwm493 mapped in same linkage group. Anderson et al. (1999) also found that Xgwm533 was associated with a QTL for scab resistance in their Sumai 3/ Stoa population.

Based on LOD scores, the linkage order of the three markers was determined to be Xgwm389-Xgwm533-Xgwm493, and the linkage distances between these markers were 5.3 cM and 4.8 cM, respectively. The three SSR markers were not polymorphic between Sumai 3 and Ning 7840. Xgwm340 was not associated with scab resistance in this population and was not analyzed further.

The major scab resistance QTL on 3BS:

Based on scab resistance evaluation data in F_5, F_6, F_7, and F_{10} generations, Xgwm533, Xgwm389, and Xgwm493 were associated with scab resistance. The allelic substitution effect ranged from 25% to 43% for PSS (percentage of scabbed spikelets) and from 2.53 to 4.72 for AUDPC (area under disease progress curve). In all four generations, Xgwm533 provided the greatest differences between groups of resistant and susceptible lines. All three SSR markers showed relatively high R^2 values when scab data from the four generations were analyzed. The phenotypic data were collected in the greenhouse using needle inoculation, therefore, the three SSR markers were associated with Type II scab resistance.

In the F_{10}, Xgwm389, Xgwm533, and Xgwm493 explained 36%, 44%, and 34% of the phenotypic variation for scab resistance, respectively. Marker Xgwm533 was associated more closely with the scab resistance QTL than the other two markers.

Physical sub-arm mapping of the major scab resistance QTL on 3BS

The three markers were analyzed on 8 Chinese Spring deletion lines, 3BS-1, 2, 3, 4, 5, 7, 8, and 9. Based on presence or absence of each SSR marker on these deletion lines, the sub-arm physical location can be identified for these SSR markers. Xgwm389 amplified the same size band in Chinese Spring, 3BS ditelosomic line, Ning 7840, and Sumai 3, but this band was not detected in any of the eight deletion lines or the 3BL ditelosomic line. Therefore, Xgwm389 is located distal to breakpoint 3BS-3 (Figure 1). For SSR Xgwm533, only one band was detected in Chinese Spring and its' 3BS ditelosomic line. This band is the same size as the polymorphic band between Ning 7840 and Clark. This band was found only in deletion line 3BS-3, and it was not found in other deletion lines of 3BS. The polymorphic band between Ning 7840 and Clark amplified by Xgwm493 also was detected only in the 3BS-3 deletion line. According to these results, Xgwm533 and Xgwm493 are located between breakpoint-3 and breakpoint-8. The physical mapping of the three markers confirmed that the map order of the three markers is Xgwm389, Xgwm533, and Xgwm493.

The SSR markers were integrated into the AFLP linkage group including the major scab resistance QTL reported previously (Bai et al, 1999) (Figure 1). Relative positions of the three SSR markers were used to determine the orientation of the linkage map. In single-factor analysis of the F_{10} AUDPC data, AFLP markers GCTG/CGAC1 and ACT/TGC7 explained 49% and 48% of the phenotypic variation, respectively (Bai et al., 1999). These two markers flanked the peak region of the major QTL for scab resistance. In the integrated map, these two markers were flanked by Xgwm533 and Xgwm389. Therefore, the major QTL is also flanked by the two SSR markers. As both Xgwm533 and Xgwm493 were located between breakpoints 3BS-8 and 3BS-3, it is clear that the major QTL is located distally to breakpoint 3BS-8. The fraction length of deletion 3BS-8 is 0.78 (Endo and Gill, 1996). It is still not clear if the major QTL is located between 3BS-3 and 3BS-8, or distal to 3BS-3.

The two SSR markers, Xgwm389 and Xgwm493, which are flanking a major QTL for scab resistance, can be used directly for MAS. The linkage distance between these two markers is 10.1 cM, and Xgwm533 is located almost between them. Xgwm533 is significantly closer to the QTL than Xgwm493 and Xgwm389. If we suppose that the QTL is located in the middle of the two markers that are 10 cM apart, the probability of missing the major QTL by selecting both two markers is 0.25%. These SSRs can be detected by nonradioactive gel electrophoresis of PCR products. We analyzed each of the SSR markers twice on the mapping population. They showed high stability and repeatability. Therefore, these markers are suitable for large scale screening of breeding populations for scab resistance when polymorphism occurs between the parents. We are validating the linkage between the three SSR markers and the major scab resistance QTL and its genetic effect in different backgrounds. We are mapping other QTL with SSRs in the same population and developing other PCR based markers for all these QTL.

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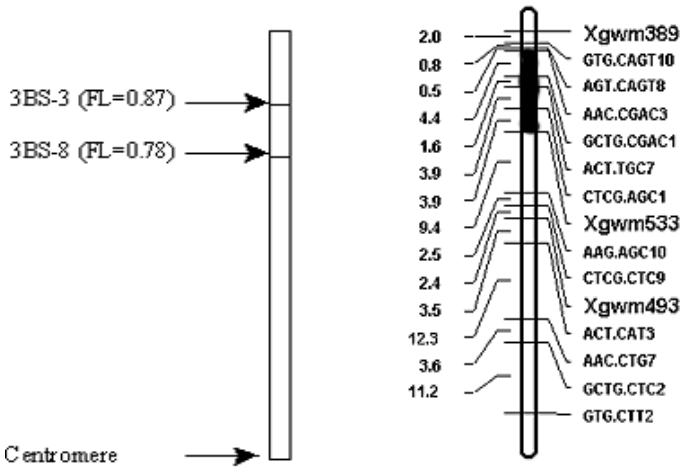

Figure1. Left: An idiogram of chromosome 3BS, arrows label breakpoints of deletion lines 3BS-3, 3BS-8 and centromere. FL means fraction length.

Right: An integrated map for microsatellite markers on chromosome 3BS and AFLP markers analyzed on the same population of Ning 7840/ Clark; distance unit between markers is cM. Dotted lines show the physical location of three microsatellite markers. Blocked bar shows putative region including the major QTL.