GENE DISCOVERY
AND
ENGINEERING RESISTANCE
A RAPID ASSAY FOR SYNTHETIC SIRNA ACTIVITY AGAINST TRI5

Thomas Baldwin and Phil Bregitzer*

ABSTRACT

Host Induced Gene Silencing (HIGS) has been demonstrated in multiple plant species as an effective and novel type of resistance to pathogens. This system functions via RNA interference (RNAi), which is initiated by double stranded RNA (dsRNA). RNAi can be initiated by Dicer-mediated production of siRNA 21mers derived from the dsRNA, or siRNA can be synthesized. Certain species of siRNA that are homologous to target pathogen gene sequences can effectively suppress gene expression. Hundreds of possible 21mers can be derived from a long dsRNA, or single siRNAs can be designed based on conserved characteristics. Here we directly test synthesized 21mer siRNA silencing capability against GFP induction driven by the TRI5 promoter in Fusarium graminearum strain TRI5prom:GFP. GFP fluorescence can be used to determine function of the trichocethene-producing pathway via induction of TRI5. This strain also constitutively expresses RFP. The best induction of TRI5 occurred with TBI media containing putricine. GFP fluorescence peaked at 44 h post- inoculation with 30,626 +/- 6165 RFU then dropped to a moderate level of expression at 54:00 h (8,422 +/- 1,491 RFU). Peak OD600 and RFP—both measures of fungal growth—were observed at 60:00 and 74:00, respectively. This system is the basis for our method of measuring down-regulation of TRI5 via direct exposure to siRNAs designed against TRI6 and TRI10, both transcription factors shown to induce TRI5.

ACKNOWLEDGEMENT AND DISCLAIMER

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TESTING TRANSGENIC SPRING WHEAT AND BARLEY LINES FOR REACTION TO FUSARIUM HEAD BLIGHT:
2014 FIELD NURSERY REPORT
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ABSTRACT

The 2014 field screening nursery consisted of 49 wheat and 11 barley entries evaluated in side by side experiments. Entries within each species experiment were arranged in a randomized complete block design with four replications in a field located at UMore Park, Rosemount MN. Trial entries and untransformed controls were submitted by the University of Minnesota (39 wheat lines + Bobwhite*, CB037* and Rollag*), Rutgers University (9 wheat lines + Bobwhite*) and the USDA (7 barley lines + Conlon and ND20448*). Lines with known reactions to Fusarium head blight (FHB) were also included as checks. The wheat checks included were the moderately resistant cultivars Alsen, RB07, Rollag and Sumai 3 and the susceptible cultivar Wheaton. The barley checks were the moderately resistant cultivar Quest and the susceptible cultivar Robust. Individual plots were 2.43 m long single rows. The trial was planted on June 6, 2014. All plots were inoculated twice. The first inoculation was applied at anthesis for wheat (July 16-July 29) and at head emergence (July 21-July 25) for barley. The second inoculation was applied three days after the initial inoculation (d.a.i.) for each plot. The inoculum was a composite of 39 F. graminearum isolates at a concentration of 100,000 macroconidia ml⁻¹ with Tween 20 (polysorbate) added at 2.5 ml L⁻¹ as a wetting agent. The inoculum was applied using a CO₂-powered backpack sprayer fitted with a SS8003 TeeJet spray nozzle with an output of 10 ml sec⁻¹ at a working pressure of 275 kPa. Mist-irrigation was applied from the first inoculation on July 16 through August 14 to facilitate FHB development. FHB incidence and severity were assessed visually 22-27 d.a.i. for wheat and 20-23 d.a.i. for barley on 20 arbitrarily selected heads per plot. FHB incidence was determined by the percentage of spikes with visually symptomatic spikelets of the 20 heads observed. FHB severity was determined as the percentage symptomatic spikelets of the total of all spikelets observed. Plots were hand harvested at maturity on September 9 (UMN wheat & barley) and September 16 (Rutgers wheat). Approximately sixty heads where harvested from each plot, threshed and the seed cleaned manually. The wheat grain was used to determine the percentage of visually scabby kernels (VSK) and then all samples (wheat and barley) were ground and submitted for deoxynivalenol (DON) analysis. In 2014, the disease severities were generally higher than the 2013 nursery. Mean FHB severities for the untransformed wheat checks, Bobwhite, CB037 and Rollag were 63, 34 and 22%, respectively. Mean FHB severities for the standard wheat checks, Alsen, Sumai 3 and Wheaton were 26, 18 and 83%, respectively. For barley, the untransformed check variety Conlon had a mean FHB severity of 22%. The barley standard checks, Quest and Stander had mean FHB severities of 17 and 34%, respectively. For the wheat entries in Bobwhite, CB037 and Rollag backgrounds, the
FHB severity data indicated that resistance was significantly expressed (P<0.05) in some transformed lines compared to the untransformed check. Similarly the FHB severities of several barley entries appeared to be statistically better than the corresponding untransformed check. The harvested grain is currently being analyzed for DON, though the data are not yet available they will be included in the poster presented at the forum.

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CHROMOSOME ENGINEERING AND NEXT GENERATION SEQUENCING ASSISTED TRANSFER AND DEPLOYMENT OF ALIEN GRASS SPECIES RESISTANCE TO FHB IN WHEAT

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ABSTRACT

The successful transfer of effective resistance to FHB from the perennial grass Elymus tsukushiensis Honda (2n=6x=42, S’tS’tH’tH’tY’tY’t, syn. Roegneria kamoi C. Koch) into wheat is the culmination of a research effort that began with the funding of a project by the McKnight Foundation between Nanjing Agricultural University (NAU) and Kansas State University (KSU) in 1995. In the 1990s, NAU scientists reported on the production of wheat x E. tsukushiensis hybrids and the disomic addition (DA) lines 1E’tS1 and its derivative TWL·1E’tS1S with effective resistance to FHB in greenhouse and field nurseries in China. The TWL·1E’tS1S chromosome consists of the short arm of chromosome 1E’tS1 joined to an unknown chromosome arm of wheat at the centromere. We crossed DATWL·1E’tS1S with the ph1ph1 (a mutant of Ph1 gene that allows pairing between wheat and alien chromosomes) stock of wheat and identified plant progenies that were homozygous ph1ph1 and carried one copy of TWL·1E’tS1S. In these plants, we expected the 1E’tS1 arm of TWL·1E’tS1S to pair randomly with one of the short arms of the group-1, wheat chromosomes 1A, 1B or 1D. To monitor recombination, we designed primers from 50 ESTs mapped to group-1S arms of wheat. One proximal (BF202643/HaeIII) and one distal (BE591682/HaeIII) EST-STS polymorphic markers were identified. We screened 488 progenies using the EST-STS marker and identified one proximal (#74) and one distal (#107) recombinant. To further characterize the recombinant chromosomes, we designed 20 SNPs using 1AS, comparative sequence analysis, and KASParTM marker assays. Recombinant #74 carried the unidentified wheat arm from TWL·1E’tS1S and proved to be agronomically undesirable. The distal recombinant #107 involved chromosome 1A of wheat, where the distal tip of 1AS was substituted by a homoeologous segment from 1E’tS1S of TWL·1E’tS1S and was designated as T1AL·1AS-1E’tS1S. Plants where T1AL·1AS-1E’tS1S substituted for chromosome 1A of wheat were fully fertile. Recombinant #107, together with susceptible (Overley) and moderately susceptible (Everest, Chinese Spring and Karl 92) controls, was screened in greenhouse tests for FHB resistance using a single-point inoculation method. The FHB index ratings of recombinant #107 carrier progenies ranged from 4.2 to 13.3%, compared to 31.7–42.5% for noncarrier progenies from the same cross, similar to the susceptible controls. The gene symbol Fhb6 has been assigned to designate this source of resistance. We developed two KASPar SNP markers to monitor the introgression of Fhb6 into adapted wheats. The material has been released as KS14WGRC61 (W–ELTSU T1AL·1AS-1E’tS1S (TA5655//CS ph1B MUT (TA3809)*2//FULLER*2 F3)) and distributed to breeders. Further prebreeding and evaluation of Fhb6 wheat lines in field nurseries is underway. (This research was supported by a grant from the USBWSI.)
RNA-SEQ CHARACTERIZATION OF TWO BARLEY FUSARIUM HEAD BLIGHT RESISTANT QTL
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ABSTRACT

Fusarium species cause Fusarium head blight (FHB) disease in wheat and barley. Resistance to the disease is controlled by quantitative trait loci (QTL). Previous QTL mapping studies in barley have identified two QTLs located on chromosome 2H bin8 and 6H bin7, respectively. To gain an understanding of the molecular mechanisms of FHB resistance, near isogenic line (NIL) pairs with contrasting QTL alleles for the 2H bin8 and 6H bin7 were developed and examined using RNA-sequencing. The transcriptomic changes of both NIL pairs were examined at 48 and 96 hours after Fusarium or mock inoculation. The host response to infection differed dramatically from 48 hours after inoculation (hai) to 96 hai. Comparative analysis of defense responses of the 2hb8 NIL pair revealed that the resistant (R) NIL exhibited broad and constitutive defense responses when compared with the susceptible (S) NIL. Cellulose synthases, UDP glycosyltransferases, cytochrome P450 enzymes, pectinesterase inhibitors, cytokinin signaling components, peptidases and lipid transfer proteins are enriched in the 2hb8 R NIL defense responses. A pair of cysteine rich receptor-like kinases were identified as promising candidate genes for the 2hb8 QTL. The 6hb7 R NIL displayed a more rapid induction of a set of defense genes than the S NIL at 48 hai and the transcript expression difference between the R and S NIL diminished at 96 hai, indicating that the R allele at the 6hb7 QTL responds more rapidly to infection. Overlap of differentially accumulated genes was identified between the two NIL pairs at 48 hai, suggesting that certain resistance mechanisms are co-regulated by the two QTL, including the DON-inactivating HvUGT13248 gene. Long noncoding RNAs (lncRNAs) have emerged as key regulators of transcription. A total of 10,338 lncRNAs were identified from our barley spike samples, among which 486 were FHB responsive. Examples of co-induction of lncRNAs and their neighboring transcripts were identified. The current transcriptomic analysis of two barley FHB QTL NIL pairs revealed the dynamics of host response to Fusarium infection and identified genes and lncRNAs that are associated with FHB resistance.
FUSARIUM CONTROL BY HOST-INDUCED GENE SILENCING

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ABSTRACT

Fusarium Head Blight (FHB) and Seedling Blight (FSB), which is caused by mycotoxin-producing fungi of the genus Fusarium, is an economically important crop disease. We assessed the potential of host-induced gene silencing (HIGS) targeting the three fungal cytochrome P450 lanosterol C-14α-demethylase (CYP51) genes, which are essential for ergosterol biosynthesis, to restrict fungal infection. In vitro feeding of CYP3RNA, a 791 nucleotides (nt) dsRNA complementary to all three paralogs CYP51A, CYP51B, and CYP51C, resulted in growth inhibition (half maximum growth inhibition [IC50] = 0.9 nM) as well as altered fungal morphology, similar to that observed after treatment with the azole fungicide tebuconazole, for which CYP51 is a target. This inhibition of fungal growth correlated with in fungus production of siRNAs corresponding to the targeted CYP51 sequences. Expression of the same dsRNA in Arabidopsis and barley rendered susceptible plants highly resistant to fungal infection. Microscopic analysis revealed that mycelium formation on CYP3RNA-expressing leaves was restricted to the inoculation sites, and that inoculated barley caryopses were virtually free of fungal hyphae. This inhibition of fungal growth correlated with in planta production of siRNAs corresponding to the targeted CYP51 sequences, as well as highly efficient silencing of the fungal CYP51 genes. The high efficiency of fungal inhibition suggests that HIGS targeting of the CYP51 genes is an alternative to chemical treatments for the control of devastating fungal diseases such as FHB and FSB.
TRANSGENIC WHEAT AND BARLEY CARRYING A BARLEY UDP-GLUCOSYLTRANSFERASE EXHIBIT HIGH LEVELS OF FUSARIUM HEAD BLIGHT RESISTANCE

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ABSTRACT

Fusarium head blight (FHB) is an old yet unsolved problem of cereal crops, mainly caused by the fungal pathogen Fusarium graminearum. During infection, trichothecenes produced by Fusarium increase fungal virulence and decrease grain quality. Previous work identified a barley UDP-glucosyltransferase gene (HvUGT13248) that detoxifies deoxynivalenol (DON) by the conversion to DON-3-O-glucoside (D3G) in transgenic yeast and Arabidopsis. Here we report successful development of transgenic wheat and barley overexpressing HvUGT13248 gene. The transgenic wheat show high levels of FHB type II resistance in the greenhouse point inoculation tests. The FHB severity of the transgenic lines were reduced by up to 91% compared to untransformed lines. We also tested these transgenic wheat in inoculated (spray inoculated with macroconidia) and mist-irrigated field experiments in three consecutive years, and they also show high levels of FHB resistance. Moreover, transgenic wheat carrying HvUGT13248 converted DON to D3G more rapidly than untransformed plants, and there was also reduced DON accumulation in the grains of the transgenic wheat harvested from the field tests. To screen wheat and barley resistance to trichothecenes, we developed a fast and convenient method by monitoring root growth of seedlings on trichothecene-containing growth media. We used this root assay to show that transgenic barley overexpressing HvUGT13248 exhibit resistance to DON. We also introduced the HvUGT13248 transgene into the elite wheat cultivar Rollag, and the backcross-derived lines exhibited high levels of FHB resistance in the greenhouse and field tests, however, the FHB severity levels were only slightly reduced from Rollag. This result suggests that the FHB resistance mechanisms provided by the Fhb1 QTL and the HvUGT13248 transgene may overlap.

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TWO NOVEL NON-SPECIFIC LIPID TRANSFER PROTEINS PROVIDE ENHANCED RESISTANCE TO A TRICHOThECENE MYCOTOXIN BY REDUCING OXIDATIVE STRESS

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ABSTRACT

Fusarium head blight (FHB) caused by Fusarium graminearum is one of the most important cereal diseases worldwide. Trichothecene mycotoxins, which are produced during infection accumulate in the grain, posing a significant health threat to humans and animals. To identify genes that improve resistance to trichothecenes, we screened an activation tagged Arabidopsis population against trichothecin (Tcin), a type B trichothecene in the same class as deoxynivalenol (DON). One of the resistant lines identified contained an activation tag upstream of two nonspecific lipid transfer protein (nsLTP) genes, AtLTP4.4 and AtLTP4.5. Expression of both nsLTP genes were induced in the mutant over 10-fold relative to wild type. Overexpression of either nsLTP gene conferred resistance to Tcin in Arabidopsis and in Saccharomyces cerevisiae. In both systems AtLTP4.4 provided greater resistance than AtLTP4.5 relative to wild type and vector transformed lines. In Arabidopsis and yeast, Tcin treatment increased reactive oxygen species (ROS) accumulation and overexpression of AtLTP4.4 attenuated ROS levels relative to the controls. Exogenous addition of GSH and other antioxidants enhanced resistance to Tcin while the addition of buthionine sulfoximine (BSO), an inhibitor of GSH synthesis, increased sensitivity to the toxin, indicating that oxidative stress contributes to trichothecene sensitivity. To further evaluate ROS induction in plants by trichothecenes, confocal microscopy was performed using Arabidopsis and tobacco leaves infused with trichothecenes and ROS was detected by staining with 2',7'-dichlorofluorescin diacetate (H2DCF-DA), which is converted to the highly fluorescent dichlorofluorescein (DCF) when oxidized by ROS. DON and Tcin treatments revealed DCF stain that colocalized with chloroplasts, the cell wall region, and possibly the apoplast. Increasing the dosages of Tcin and DON intensified the DCF staining of chloroplasts. Overnight treatments with a high dose of DON (240 µM) released chlorophyll into the cytoplasm as observed after treatment with a low concentration of paraquat. These results demonstrate that trichothecenes target chloroplasts and induce ROS and that overexpression of a specific Arabidopsis nsLTP protects against trichothecene-induced oxidative stress possibly by increasing the antioxidant defense.
DEVELOPING TRANSGENIC WHEAT AND BARLEY THAT EXHIBIT RESISTANCE TO FUSARIUM GRAMINEARUM VIA GLUCOSIDE CONJUGATION OF TRICHOThECENE MYCOTOXINS

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ABSTRACT

Fusarium graminearum infection of wheat and barley results in production of trichothecene mycotoxins including deoxynivalenol (DON) and nivalenol (NIV). These mycotoxins result in increased fungal virulence and reduce grain quality. Numerous transcriptomic studies have been conducted by our lab on the wheat/barley – F. graminearum interaction. These studies have identified a set of genes that may provide resistance to F. graminearum infection via conjugation, degradation or transport of trichothecenes. In addition, these studies also provide an understanding of F. graminearum genes that are expressed during infection. For example, the F. graminearum transcriptome responds differently to wheat carrying either resistant or susceptible alleles for Fhb1. From these studies we identified a barley UDP-glucosyltransferase (UGT13248) that exhibited DON resistance in yeast. Transgenic wheat expressing UGT13248 exhibited a high level of type II resistance in the greenhouse and resistance in the field that approaches the level of resistance conferred by Sumai 3. The mechanism of resistance conferred by UGT13248 is via conjugation of DON to DON-3-O-glucoside. Backcross families carrying Fhb1 (type II resistance) derived from Rollag and the UGT13248 transgene were screened in the greenhouse and field. The level of resistance in plants carrying Fhb1 alone and those carrying Fhb1 and the UGT13248 transgene were similar with a few Fhb1/UGT13248 containing lines exhibiting a slight reduction in severity compared to those carrying Fhb1 alone. The lack of reduction in disease severity may be due to either (1) Fhb1 and the UGT13248 acting in the same manner or (2) the level of resistance conferred by Fhb1 is so high that it is difficult to obtain increased resistance. It is noteworthy that transgenic wheat carrying UGT13248 also exhibits type II resistance to 3-ADON- and NIV-producing strains of F. graminearum, indicating that UGT13248 acts on a wide range of trichothecene mycotoxins. Interestingly, overexpression of UGT13248 in barley resulted in resistance to DON in root assays. Overall, our results demonstrate that UGT13248 is an effective gene for conferring resistance to F. graminearum infection.
Genomic selection (GS) is a marker based selection method that promises to improve and accelerate the breeding process in plants and animals. Numerous studies have investigated the gain per unit time; however few have compared the gain from GS and phenotypic selection (PS) using empirical data. In this study, we used Fusarium head blight (FHB) severity and deoxynivalenol (DON) data from five consecutive years of selection (2006 – 2010) to compare the gain between GS and PS. In each year, about ninety six barley breeding lines were phenotypically evaluated in FHB and DON trials and that data was used to conduct PS. A set 168 parental lines, that were genotyped with 1,536 SNP markers and phenotyped for FHB and DON, were used as a training population to predict the performance of the breeding lines in each of the five years using RR-BLUP. We selected best (top 10%) breeding lines is each year using PS and GS. These lines were re-evaluated together in 4 trials in Minnesota and North Dakota to compare the gain from selection using the two selection schemes. In general, the gain from GS and PS were similar across the 5 years indicating that GS could maintain similar gains per cycle of selection, but at a reduced cost and shorter cycle time.

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ENGINEERING RESISTANCE AGAINST FUSARIUM GRAMINEARUM
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ABSTRACT

Fusarium graminearum is the principal causative agent of Fusarium head blight (FHB), a destructive disease of wheat and barley. Under laboratory conditions, F. graminearum can also cause disease on leaves and flowers of Arabidopsis thaliana, a model plant for molecular-genetic studies. Our results have shown that pathogen-associated molecular patterns (PAMPs), can stimulate PAMP-triggered immunity (PTI), which can enhance resistance against F. graminearum in Arabidopsis. Furthermore, application of a bacterial PAMP was capable of enhancing FHB resistance in wheat, thus suggesting that the PTI mechanism can potentially be targeted for enhancing resistance against F. graminearum in wheat. We have engineered one of these defense elicitors for expression in Arabidopsis and find that these plants exhibit elevated resistance to leaf and floral inoculation with F. graminearum. Transgenic wheat plants expressing the defense elicitor have been generated and are being evaluated for resistance to FHB. In addition, we have engineered wheat to constitutively express a transcription factor involved in the activation of PTI. Results obtained with these plants will be presented, in addition to other strategies for enhancing FHB resistance that are underway in our lab.

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