GENE DISCOVERY AND ENGINEERING RESISTANCE

DEVELOPMENT OF A FUSARIUM HEAD BLIGHT (FHB) RESISTANT WHEAT VIA THE OVER-ACTIVATIONS OF TWO WHEAT NATIVE TRANSGENES

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OBJECTIVES

1. Search the GeneBank, and assemble constructs.
2. Transform wheat genome via biolistic gene-gun technology
3. Perform molecular analysis to confirm the transgenes integration and their real-time transcriptions.
4. Grow plants to maturity, and inoculate spikes using Ph1 isolates.
5. Measure and analyze disease spread severity.
6. Collect and analyze data.

INTRODUCTION

The wheat (*Triticum aestivum* L.) Fusarium head blight (FHB), mostly caused by *Fusarium graminearum* has resulted in $3 billion loss in North America. This pathogen not only reduces the crop yield, but also contains, deoxynivalenol (DON), a mycotoxin that is harmful to the human and animal health (Pierson et al., 2015).

Several reports confirm the resistance of Chinese wheat lines; Suami 3 and Wangshuibai to FHB. However, strategies to transfer the FHB resistance genes into commercial wheat genotypes via conventional breeding have not been successful (Buerstmayr et al., 2009) mostly because breeding of these two genotypes with commercial wheat lines are very difficult due to the complexity of the resistance to FHB pathogen trait (Xiao et al., 2013).

Although the *Fhb1* gene was identified for 17 years (Waldron et al., 1999), its map-based cloning very recently revealed the origin of the *Fhb1* gene in wheat genome (Rewat et al., 2016).

Xiao et al. (2013) inoculated FHB-resistant Wangshuibai and another FHB-susceptible wheat landrace, and performed a series of transcriptome-based discoveries of pathways and genes associated with the FHB resistance using high-throughput RNA sequencing. They reported that a few genes including the PR5, PR14, the ABC transporter and JA signaling pathway mediated by *Fhb1* were important in FHB resistance. They also confirmed the involvement of the coronatine insensitive 1-like (*coi1*) receptor in response to infection by the FHB pathogen, and reported that a chromosome deletion in the susceptible wheat landrace might play a role in FHB susceptibility in wheat.

Here, the authors studied the overexpression of genes encoding the wheat native protein *coi1* and *tlp1* in wheat genome for resistance to FHB.

MATERIALS AND METHODS

Construct assembly: The gene constructs (pCoi1-JS101 and pTLP-JS101; Figure 1) were developed. The *pHAtlp1* constitutes the wheat native *coi1* coding sequences regulated by the rice actin1 *(Act1)* promoter and the potato protease II terminator (*Pin3*'). The pTLP-JS101 constitutes the wheat native *tlp1* regulated by the *Act1* promoter and *Pin3'*. Both, pCoi1-JS101 and pTLP-JS101 constructs harbor the *bar* herbicide resistance gene cassette.

Plant Material and Genetic Transformation: Wheat cv. Bobwhite seeds were germinated in the greenhouse, plants were grown to maturity, and their immature embryos were isolated. Then, the sterilized immature embryos were bombarded with a 1:1 ratio of a combination of the *pCoi1* and *pTLP1* (Figure 1). The bombarded immature embryos were cultured in-vitro following Zhang et al. (2000). Putative transformants were transferred to a growth chamber and tested for herbicide resistance using a leaf painting assay with a 0.1 % aqueous Liberty™ solution containing 18.9 % glufosinate ammonium (Nguyen et al., 2013), and herbicide resistant plantlets were regenerated in a greenhouse to maturity.

Molecular Confirmation: Genomic DNA was extracted from herbicide resistant plantlets, as well as from their wild-type non-transgenic plants using the CTAB method (Xin and Chin, 2012), and polymerase chain reaction (PCR) was performed following the authors previous report (Nguyen et al., 2013) to confirm the integration of each of the two transgenes in wheat genome using specific primers.

qPCR**:** Total RNA was extracted from transgenic as well as the wild-type non-transgenic plants using Omega E.Z.N.A.® Plant RNA Kit, and cDNA strands were synthesized using GoScript™ Reverse Transcriptase (Promega catalogue no. A5003). Then the real-time (q) PCR was performed using Applied biosystems Fast SYBR® Green Master Mix.

Conidia Preparation Spike Inoculation and Data Collection: The *Fusarium graminearum* Ph-1 isolates were grown on Nash-Snyder media, and conidia were collected following the standard method, quantified using a hemocytometer, and diluted to a final concentration of 1.0x105 conidia/mL. Single central floret of each spike was inoculated by injecting 10µL of the *F. graminearum* suspension of ~ 1000 conidia into each floret, and the inoculated florets were marked for latter survivability data collections. Finally, disease rating was conducted at 7, 14 and 21 days after inoculation. Disease progression was recorded for each inoculated spike by counting the healthy spikelets in both directions (above and below) from the point of inoculation. The area under the disease progress curve (AUDPC) was calculated using Shaner and Finney's (1977) equation to describe the increase in plant susceptibility during the experiment.

RESULTS AND DISCUSSIONS

Herbicide resistant lines were identified, and PCR positive herbicide resistant plants showing the highest level of the two transgenes transcriptions were identified based on the qPCR data for both *Coi1* and *trlp1* genes in T1 plants.

The spikes of inoculated plants that showed a combination of highest transcripts of both *tlp1 and Coi1* showed an impressive level of resistance against the pathogen (Figure2), with disease severity ranging only from 1 to 15%, while the wild-type control plant spikes suffered at 95%. The AUDPC (Figure 3) showed a significant difference between the wild-type versus the transgenic lines (ANOVA $P<0.01$). Statistical analysis results show that the infection of wild-type non-transgenic line ($P=0.00$-0.018) was significantly higher than all six transgenic lines.

ACKNOWLEDGEMENTS

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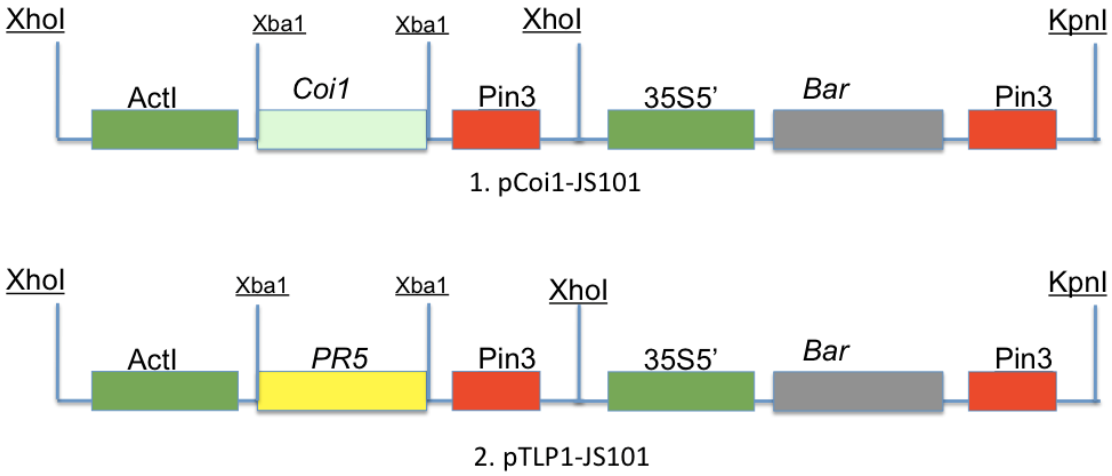

Figure 1. The pCoi1-JS101 constructs and pTLP1-JS101

Figure 2. Greenhouse level symptoms of *Fusarium graminearum* cell-free mycotoxin single spot microinjection of the wild-type non-transgenic spike (left) versus the first generation (T0) tlp1-coi1 real-time overexpressed spike (right) 21 days after inoculation. Note the site of inoculation (SOI) as single black spot on each spike.

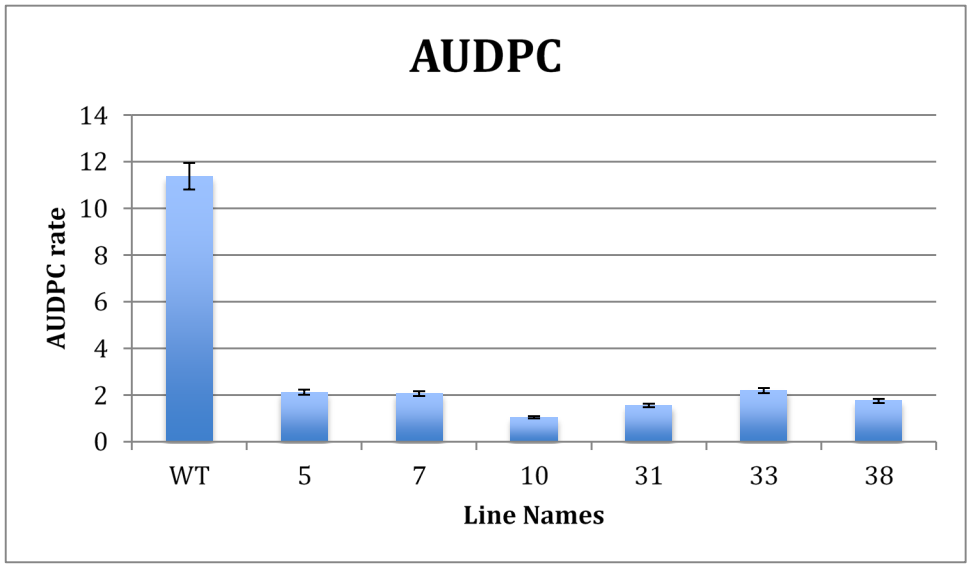

Figure 3. The area under disease progress curve (AUDPC) rates for the wild-type non-transgenic versus each of the six T1 (second generation) tlp1-Coi1 genetic lines.

INSIGHT INTO THE MECHANISM OF THE *TRI6* RNA ENTERFERENCE ABLATING DEOXYNIVALENOL PRODUCTION IN *FUSARIUM GRAMINEARUM* WITH PATTERNS OF SIRNA PRODUCTION

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ABSTRACT

Deoxynivalenol (DON) contamination of small grains caused by Fusarium head blight is a problem of economic and health importance that may be addressed by RNAi interference (RNAi)-based host-induced gene silencing (HIGS). RNAi in higher eukaryotes, including fungi, involves processing double stranded RNA (dsRNA) into small interfering RNA (siRNA) that silence genes based on homology. Six initial randomly inserted transgenic *F. graminearum* (strain PH1) mutants containing the RNAi vector pTRM-TRI6 were studied, containing a full length inverted repeat of *TRI6,* a transcription factor that positively regulates DON production. The mutant lines showed typical phenotypes of DON reduction, including reduced virulence on wheat, and reduced DON in barley infection and in non-host toxin inducing media. The sRNA populations of three mutant lines, but not PH1, had abundant siRNA species that mapped to *TRI6*, with 22 nt siRNA identified as the most abundant. The discontinuous and repeatable siRNA mapping, consistent peaks, and overrepresentation of siRNA with starting 5' uracil base demonstrated clear preferences for fungal dicer to produce specific siRNAs. Subsequent experiments, in which transformation with five shorter (200-250 nt) inverted repeats with homology to *TRI6* (each targeting a different region of *TRI6*), showed patterns of siRNA processing for each individual inverted repeat that were similar to that resulting from the processing of the corresponding section of the inverted repeat of full length *TRI6*. Dicer patterns for dsRNA processing have implications for design of efficient RNAi silencing vectors. Understanding the siRNA profiles that result from RNAi constructs is critical to optimizing RNAi applications, such as HIGS, that are designed to reduce pathogenicity and mycotoxin production in the field.

ACKNOWLEDGEMENT AND DISCLAIMER

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HOST-INDUCED SILENCING OF *FUSARIUM CULMORUM* GENES PROTECTS WHEAT FROM INFECTION

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ABSTRACT

Plants producing antisense or double-stranded RNA molecules that target specific genes of eukaryotic pests or pathogens can become protected from their attack. This beneficial effect was also reported for plant-fungus interactions and is believed to reflect uptake of the RNAs by the fungus via a yet unknown mechanism, followed by target-gene silencing. Here we report that wheat plants pre-infected with barley stripe mosaic virus (BSMV) strains containing antisense sequences against target genes of the Fusarium head blight (FHB) fungus *F. culmorum* caused a reduction of corresponding transcript levels in the pathogen and reduced disease symptoms. Stable transgenic wheat plants carrying an RNAi hairpin construct against the β-1, 3-glucan synthase gene *FcGls1* of *F. culmorum* or a triple combination of Fc*Gls1* with two additional, pre-tested target genes also showed enhanced FHB resistance in leaf- and spike inoculation assays under greenhouse- and near-field conditions, respectively. Microscopic evaluation of *F. culmorum* development in plants transiently or stably expressing Fc*Gls1*-silencing constructs revealed aberrant, swollen fungal hyphae indicating severe hyphal cell wall defects. The results propose HIGS as a plant protection approach that may also be applicable to highly FHB-susceptible wheat genotypes. To better understand whether HIGS is a natural phenomenon, small RNAs from *F.graminearum* infected barley have been sequenced and functional analysis of potential HIGS targets in *F. culmorum* is in progress.

FHB RESISTANCE GENES - GENES WITH MULTIPLE BENEFITS?

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ABSTRACT

Fusarium head blight (FHB) is an economically important disease on both barley and wheat. One of the main foci of our team is to identify cereal genes that are of benefit to breeders in terms of marker-assisted selection for FHB resistance, and at the same time, we elucidate the signaling mechanisms involved in the host-pathogen interactions. This works compliments our research that aims to identify fungal endophytes that show potential for the enhancement of crop establishment and the inhibition of diseases. Having identified candidate genes we use a combination of gene silencing and gene overexpression studies to validate their role in resistance and use protein-protein interaction and other biochemical studies to determine their mode of action. Using this pipeline we have validated the role of specific genes in FHB resistance. For example, we used a functional genomics approach to identify genes up-regulated by the *Fusarium* mycotoxin deoxynivalenol (DON) in a population segregating for toxin resistance, this trait being a component of FHB resistance. This study delineated genes potentially involved in toxin resistance and further studies on an ABC transporter (*TaABCC3.1*), a novel orphan gene (*TaFROG*) and a cytochrome P450 (*TaCYP450*) validated their role as quantitative resistance genes. At the cellular level, the TaFROG protein interacts with and enhances the activity of the central stress regulator SnRK1. It also interacts with other cellular machinery including a novel transcription factor. Ongoing studies are determining the allelic diversity of candidate FHB resistance genes and their promoters. One emerging trend is that select FHB resistance genes affect wheat yield. In small-scale glasshouse trials we observed that either gene silencing and/or overexpression of select genes affected yield and future work will determine the effect of these genes on yield in larger scale yield trials.

TRANSGENIC WHEAT LINES UPREGULATED FOR GENES IN LIGNIN BIOSYNTHESIS AS POTENTIAL RESISTANCE SOURCES AGAINST FUSARIUM HEAD BLIGHT

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ABSTRACT

The goal of this research is to identify Fusarium head blight (FHB) resistance in transgenic wheat lines expressing genes involved in the monolignol biosynthetic pathway. Monolignols are the subunits of the lignin polymer, which are secreted into cell walls to provide structural support, and this pathway also is induced upon pathogen attack. It had been previously shown that increased resistance to *Fusarium* grain pathogens and elevated phenolic metabolites could result in sorghum lines with alterations in monolignol biosynthesis. For the present research, four sorghum genes, one a MYB transcription factor (*SbMyb60*) that acts as a positive regulator, and three for genes encoding enzymes in the sorghum monolignol pathway, caffeoyl_CoA 3-*O*-methyltransferase (*SbCCoAOMT*), 4-coumarate-coenzyme A ligase, (*Sb4CL*), and *p*-coumarate 3-hydroxylase (*SbC3H*), were cloned into expression constructs and individually transformed into wheat (spring CB037), using *Agrobacterium tumefacians*-mediated transformation. Previous research with sorghum overexpressing *SbMyb60* demonstrated that this transcription factor is associated with induction of lignin biosynthesis. Immunobolt analysis of protein extracts from transformed wheat lines showed that expression of *SbMyb60* resulted in increases of the endogenous phenylalanine ammonia lyase (PAL) and 4CL protein levels. Similar analysis of transformed wheat lines expressing *SbCCoAOMT* and *Sb4CL* resulted in detectable levels of the corresponding enzymes. *SbC3H* encodes for a membrane-associated cytochrome P-450, and is difficult to detect using immunoblot analysis; therefore, reverse transcriptase quantitative PCR was conducted to measure transcript levels of this gene in transformed wheat lines. Two elite events were identified for each of the four expression vectors. The eight transgenic lines, along with untransformed CB037 and resistant and susceptible checks, are being screened for Type I (to initial infection) and Type II (to spread after infection) resistance to FHB and accumulation of trichothecene mycotoxins in the grain. Metabolite analysis also will be conducted on inoculated and control plants to determine whether altered levels of one or more phenolic metabolites can be associated with increased resistance or tolerance to FHB. In this way, genes in the monolignol biosynthesis pathway or specific secondary metabolites may be identified as potential markers for breeding for FHB resistance in wheat.

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EXPLORING FUSARIUM HEAD BLIGHT DISEASE CONTROL BY RNA INTERFERENCE

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ABSTRACT

RNA interference (RNAi) technology provides a novel tool to study gene function and plant protection strategies. *Fusarium graminearum* is the causal agent of Fusarium head blight (FHB), which reduces crop yield and quality by producing trichothecene mycotoxins including 3-acetyl deoxynivalenol (3-ADON) and 15-acetyl deoxynivalenol (15-ADON). In this study, we designed and synthesized dsRNA targeting the transcription factor *tri6*, which is a key regulator of DON biosynthesis. Wheat heads were excised, point-inoculated with *F. graminearum*, and treated with a solution of *tri6*-dsRNA or a water control. FHB spread was scored after 8 days, and wheat heads were then collected to evaluate gene expression and DON production. Our results showed that *tri6*-dsRNA reduced disease spread and DON production in infected wheat heads in comparison to water treated controls. Furthermore, the expression of tri6 was significantly reduced in *tri6*-dsRNA treated wheat heads. Our study suggests that dsRNA application is a promising strategy for plant disease control. Further investigation will be focused on identifying the most effective dsRNA target and optimizing efficient delivery methods.

CHARACTERIZATION OF SMALL RNAS FROM *FUSARIUM*-INOCULATED BARLEY SPIKE TISSUES

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ABSTRACT

Fusarium species cause Fusarium head blight (FHB) disease in wheat and barley around the world. Transcriptomic analyses of barley-*Fusarium* interaction have revealed complex molecular mechanisms associated with disease resistance. Previously we analyzed the gene expression profiles of a near-isogenic line carrying a 2Hb8 QTL (2Hb8 R NIL) for FHB and its recurrent parent M69 using RNA-Seq. In this study, deep sequencing of small RNAs from infected and non-infected spike tissues of the same lines was performed. Small RNAs, including microRNAs (miRNAs), have regulatory functions in diverse biological processes such as development and response to environment. Twenty-four sRNA libraries were sequenced with the illumina platform and the average read counts were 12M per library. Mature miRNA reads were mapped to 36 barley miRNA families and 38 homologous miRNA families. Novel miRNAs were predicted using the miRDeep2 package. Overall, the total read count of miRNAs tended to decrease after infection in the susceptible genotype while the read count remained stable in the R NIL. At 96 hour after inoculation, the R NIL exhibited higher expression of miRNAs when compared to the susceptible recurrent parent. Targets of conserved barley miRNA families will be predicted using psTarget and the results will be presented.

A BARLEY UDP-GLUCOSYLTRANSFERASE PROVIDES RESISTANCE TO NIVALENOL AND NIVALENOL-PRODUCING *FUSARIUM GRAMINEARUM*

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ABSTRACT

Fusarium head blight (FHB) is a cereal disease that causes severe yield losses and mycotoxin contamination of small grains. The main causal pathogen, *Fusarium graminearum,* produces trichothecenes mycotoxins, such as deoxynivalenol (DON) or nivalenol (NIV). Nivalenol-producing *Fusarium* strains have been identified in North America and although not currently a major issue, could pose a potential future problem. A barley UDP-glucosyltransferase, *HvUGT13248*, was previously identified that efficiently detoxifies DON to the less toxic DON-3-*O*-β-D-glucoside and provides a high level of FHB resistance in transgenic wheat. Here we report that HvUGT13248 also converts nivalenol into nivalenol-3-*O*-β-D-glucoside, a much less toxic derivative. Interestingly, HvUGT13248 exhibits higher affinity and enzymatic activity for NIV than DON. Overexpression of *HvUGT13248* leads to increased nivalenol resistance in yeast and *Arabidopsis thaliana*. Also, transgenic wheat overexpressing *HvUGT13248* exhibit enhanced ability to detoxify NIV and high levels of type II resistance to a nivalenol-producing *Fusarium graminearum*. Taken together, our results demonstrate that *HvUGT13248* exhibits resistance to both DON and NIV.

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CHARACTERIZATION OF A GENUS SPECIFIC UNIDENTIFIED OPEN READING FRAME FOUND WITHIN THE MITOCHONDRIAL GENOME OF *FUSARIUM*

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ABSTRACT

Sequencing and annotation of the mitochondrial DNAs (mtDNAs) in species of the filamentous fungal genus *Fusarium* identified a highly variable region (HVR) located between the *rnl* and *nad2* genes. Prior characterization of this region identified the presence of a large unidentified open reading frame (LV-uORF) found in at least one strain of all species characterized to date. The predicted polypeptides of these LV-uORFs are variable in size, but in most cases are highly conserved within a species complex. Analysis of the HVR of 32 isolates from four species within the *Fusarium graminearum* Species Complex (FGSC) detected a highly conserved putative polypeptide of 1931 amino acids (ORF1931). The LV-uORF is actively transcribed, but the putative polypeptide (1931p) has yet to be detected. Current research aims to identify and localize 1931p within *F. graminearum* PH-1 mitochondria via immunoblotting and subcellular fractionation. Interestingly, isolates from the *Fusarium oxysporum* Species Complex (FOSC) containing the LV-uORF exhibit greater variability, ranging from 2200 to 2500 amino acids, with other isolates lacking the ORF entirely. As such, the FOSC is an ideal lineage for both phylogenetic and functional analysis to determine the function of this putative gene product. Here we examine phylogenetic evidence of the allelic diversity found within the LV-uORF of a single subspecies of the FOSC.

ANTIFUNGAL PLANT DEFENSINS: MECHANISMS OF ACTION AND ENGINEERING DISEASE RESISTANT CROPS

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ABSTRACT

Host defense peptides have evolved in plants to protect from the damaging effects of fungal pathogens. Defensins are sequence divergent cysteine-rich antifungal peptides of innate immunity expressed in all plants. They exhibit potent antifungal activity *in vitro* and therefore have potential for use in transgenic crops for fungal disease resistance. MtDef4 and MtDef5 are two sequence-divergent apoplast-localized defensins expressed in *Medicago truncatula*. MtDef4 is a monomeric defensin of 47 amino acids, whereas MtDef5 is a novel dimeric defensin containing two monomeric defensin peptides A & B joined by a 7-amino acid linker. Like all previously characterized monomeric plant defensins, MtDef4 inhibits the growth of filamentous fungi including *Fusarium graminearum* at micromolar concentrations. In contrast, dimeric MtDef5 inhibits the growth of these fungi at nanomolar concentrations. MtDef4 and MtDef5 rapidly permeabilize the plasma membrane of *F. graminearum* and translocate into the cytoplasm of this fungus. These defensins differ from each other in sequence, net charge and hydrophobicity. The mode-of-action studies have revealed that they exhibit different modes of antifungal action.

Transgenic wheat lines expressing apoplast-targeted MtDef4 exhibit strong resistance to an obligate biotroph *Puccinia triticina*, causal agent of an economically important leaf rust disease. Histopathological analysis suggested the presence of both pre- and posthaustorial resistance to leaf rust in these transgenic lines. MtDef4 did not, however, affect the root colonization of a beneficial arbuscular mycorrhizal fungus *Rhizophagus irregularis*. This study demonstrates that the expression of apoplast-targeted plant defensin MtDef4.2 can provide substantial resistance to an economically important leaf rust disease in transgenic wheat without negatively impacting its symbiotic relationship with the beneficial mycorrhizal fungus.

EXPRESSION OF BEAN *PGIP2* UNDER CONTROL OF THE BARLEY *LEM1* PROMOTER LIMITS *FUSARIUM GRAMINEARUM* INFECTION IN WHEAT

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ABSTRACT

Fusarium Head Blight (FHB) caused by *Fusarium graminearum* is one of the most destructive fungal diseases of wheat worldwide. The pathogen infects the spike at flowering time and causes severe yield losses, deterioration of grain quality, and accumulation of mycotoxins. Better understanding of the means of pathogen entry and colonization of floral tissue is crucial to providing effective protection against FHB. Polygalacturonase inhibiting proteins (PGIPs) are cell wall proteins that inhibit the activity of polygalacturonases (PGs), a class of pectin-depolymerizing enzymes secreted by microbial pathogens, including *Fusaria*. The constitutive expression of a bean PGIP (PvPGIP2) under control of the maize *Ubi1* promoter limits FHB symptoms and reduces mycotoxin accumulation in wheat grain [Janni et al. 2008 Molec. Plant Microb. Interact. 21:171]. To better understand which spike tissues play major roles in limiting *F. graminearum* infection, we explored the use of PvPGIP2 to defend specific spike tissues by expressing it under control of the barley *Lem1* promoter [Somleva and Blechl 2005 Cer. Res. Comm. 33:665]. We show here that the expression of PvPGIP2 in lemma, palea, rachis and anthers reduced FHB symptoms caused by *F. graminearum* compared to symptoms in infected nontransgenic plants. However, the expression of PvPGIP2 only in the endosperm under control of a HMW-glutenin gene promoter did not affect FHB symptom development, indicating that once the pathogen has reached the endosperm, inhibition of the pathogen's PG activity is not effective in preventing its further spread.