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Enhanced Pest Resistance of Maize Leaves Expressing Monocot Crop Plant-Derived Ribosome-Inactivating Protein and Agglutinin

Patrick F. Dowd,*[,†] Eric T. Johnson,[†] and Neil P. Price[‡]

[†]Crop Bioprotection Research Unit and [‡]Renewable Product Technology Research Unit, National Center for Agricultural Utilization Research, Agricultural Research Service, U.S. Department of Agriculture, 1815 North University Street, Peoria, Illinois 61604, United States

ABSTRACT: Although many insect resistance genes have been identified, the number of studies examining their effects in combination using transgenic systems is limited. This study introduced a construct into maize containing the coding sequence for maize ribosome-inactivating protein (MRIP) and wheat germ agglutinin (WGA). Many transformants produced both the MRIP and WGA in leaves. Mature leaves expressing higher levels of these two proteins were more resistant to feeding by first-instar larvae of fall armyworms (*Spodoptera frugiperda*) and corn earworms (*Helicoverpa zea*), and the level of resistance was correlated with levels of MRIP and WGA. There was also some indication that resistance to *Fusarium verticillioides* was increased in the transgenic plant leaves. No statistically significant synergism or antagonism occurred between the activities of the two proteins. MRIP and WGA represent compatible class examples of food plant-derived proteins for multigene resistance to insects.

KEYWORDS: *ribosome-inactivating protein, insect resistance, agglutinin, Helicoverpa, Spodoptera*

INTRODUCTION

Insect damage is responsible for an estimated $400 billion loss in field crops worldwide each year.[1] Maize is one of the world's major human and animal food grain crops and is also used in ethanol production. Prior to the introduction of transgene resistance to some insect pests, maize losses to insects in the United States were approximately $1.5 billion annually.[2] In addition, insect kernel damage can facilitate the production of mycotoxins, which are toxic to humans and animals, and result in estimated direct and indirect losses of hundreds of millions of dollars.[3,4]

The increases of insect resistance in maize through breeding or genetic engineering has greatly reduced losses to some insect species, mainly through transgenic expression of the *Bacillus thuringiensis* (Bt) crystal protein gene over the past 10 years. However, movement and introduction of new pest species or genetic variants of pests (i.e., western bean cutworm, *Loxagrotis albicosta*, western corn rootworm, *Diabrotic virgifera virgifera*, trichothecene-producing *Fusarium graminearum* in the Americas) continues to make insect and disease control in maize a challenge. The recent development of insect populations resistant to Bt expressed in different crops has led to the removal of some Bt varieties from sale.[5−8] All of these issues have further stimulated investigations of alternatives to Bt, especially those that are derived from food plants (preferably self-derived), as plant-derived genes are less objectionable to consumers.[9] Combinations of plant-derived resistance genes are likely to result in more durable resistance, but there is little information on how multiple insect resistance genes may interact; combinations may be antagonistic, additive, or synergistic.

Most prior investigations of gene combinations have concentrated on combinations of microbial chitinases and other proteins such as the Bt protein; presumably chitinases help increase penetration of toxic molecules to the target site by dissolving the peritrophic membrane barrier in the insect midgut.[10] However, no food plant-derived chitinases active against caterpillars have been reported. Other combinations of insect resistance genes have been less commonly investigated in transgenic systems, and few have come from monocot crop plants. One potential candidate protein for insect resistance to maize pests is the maize ribosome-inactivating protein (MRIP), which is found in mature kernels and cleaves part of the ribosome.[11] Expression of this protein in leaves of different species of plants has resulted in increased resistance to both insects[12] and fungi.[13] Another food crop protein that has anti-insect[14] and antifungal activities[15] is wheat germ agglutinin (WGA), which appears to affect insects by disrupting the peritrophic membrane.[16] Although its allergenicity to some people makes WGA less practical from a commercial standpoint, WGA provides a model type of protein within that class which can be used to investigate in combination with MRIP. Here we report the result of investigations in which these two proteins were transgenically expressed in maize leaves and increased resistance two major maize insect pests, the corn earworm (*Helicoverpa zea*) and fall armyworm (*Spodoptera frugiperda*), as well as the fungus *Fusarium verticillioides*, which produces mycotoxins in maize.

MATERIALS AND METHODS

Insects. Corn earworms and fall armyworms were reared on a pinto bean diet at 27 ± 1 °C and 50 ± 10% relative humidity with a 14:10 h light/dark photoperiod as described previously.[17] Both species were originally collected from maize near Champaign, IL, USA, and Ames, IA, USA, and

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have been in culture over 20 years without additional field-collected supplementation. First-instar larvae were used in assays.

Fungi. The *F. verticillioides* strain AMRF-1 was originally isolated by R. H. Proctor (USDA-ARS, NCAUR, Peoria, IL, USA) from popcorn collected in Illinois in 2009. Cultures were grown on V8-agar at 27 ± 1 °C until sporulation occurred. The culture was flooded with 5 mL per plate of 0.01% Triton X 100 to yield a spore stock, which was quantitated by dilution plating to yield 1×10^7 colony-forming units (cfus) per mL.

Plant Growth. Regenerated plantlets were transplanted and grown in soil type 3.[18] The lower half of the 8 L pots was filled with SB330 bark mix (Sungro Horticulture, Bellevue, WA, USA) containing 250 mL of Osmocote 14:14:14 (Scotts Grace Sierra, Milpitas, CA, USA), 125 mL of a 44 mL/L stock of Sprint 330 iron chelate micronutrient solution (Becker Underwood, Ames, IL, USA), and 1 L of a 10 mL/L liquid limestone solution (Limestone F, Cleary Chemical Corp., Dayton, NJ, USA). The upper half of the pots was filled with RediEarth (Scotts Grace Sierra). Pots were watered daily with deionized water. Plantlets transplanted to pots were acclimated under plastic cups with cloth vents for a few days. Plants were maintained in a plant growth facility under an 18 ± 2 °C day and 24 ± 2 °C night temperature regimen at 50 ± 10% relative humidity with a 16:10 h light/dark photoperiod, as described previously.[18]

Construct Preparation. Candidate genes for transformation were obtained from multiple sources. The MRIP construct, MOD1,[11] was a generous gift of R. S. Boston, Department of Botany, North Carolina State University. The wheat germ agglutinin isolectin D (WGA) (GenBank M83926) was a generous gift of N. V. Raikhel, Department of Botany, University of California, Riverside. The tobacco hornworm (*Manduca sexta*) chitinase (THWC) (GenBank U02270) was a generous gift of S. Muthukrishnan, Department of Biochemistry, Kansas State University.

Each transgene was amplified from its host plasmid using PfuUltra High Fidelity DNA polymerase according to the manufacturer's instructions (Stratagene, La Jolla, CA, USA) so that the nucleotides GCC immediately preceded the first ATG of the coding sequence, as previously recommended.[19] In addition, appropriate restriction enzyme sequences were included in the primers to facilitate cloning. Standard molecular biology techniques[20] were used to develop the three host plasmid constructs. The cauliflower mosaic virus (CMV) 35S enhancer and basal promoter of the maize C4PPDK gene (HBT promoter)[21] were placed ahead of the MRIP gene (that had the N-terminal histidine tag and enterokinase sequence of MOD1 replaced with a single methionine prior to introduction) into plasmid pSK4. Sequencing indicated an additional codon was present near the terminal portion of the sequence that is cleaved off during activation (AAATAASADNDDDEA instead of CATAASADNDDDEA). The maize ubiquitin promoter was placed ahead of the WGA in plasmid pKS2-3. The CaMV 35S promoter and maize Adh1 intron 1 were placed ahead of the tobacco hornworm chitinase gene in the plasmid pKS221. The NOS (*Agrobacterium tumefaciens* nopalene synthase) terminator followed each promoter gene combination. All constructs were sequenced using a Big Dye Terminator Cycle Sequencing Kit v. 3.1 (ABI, Foster City, CA, USA) to ensure no sequence changes had occurred during construction. The three different host plasmids contained unique recombination sites that facilitated transfer into one Gateway vector.[22] The three gene expression cassettes were cloned into maize transformation vector pTF101.1gw3 using Gateway LR Clonase I PLUS, according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA), with the order of candidate genes being MRIP-WGA-THWC (Figure 1).

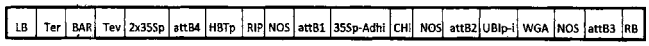

Figure 1. Map of multigene construct used to introduce maize ribosome-inactivating protein (MRIP), tobacco hornworm chitinase (THWC), and wheat germ agglutinin (WGA): LB and RB, left and right borders, respectively, for T-DNA insertion; Ter, transcription terminator for BAR gene; BAR, selectable marker gene; Tev, tobacco etch virus translation enhancer; 2×35Sp, two copies of 35S cauliflower mosaic virus promoter; attB(1−4), recombination sites used for Gateway cloning; HBTp, HBT promoter; RIP, maize ribosome-inactivating protein; NOS, nopaline synthase gene terminator from *Agrobacterium tumefaciens*; 35Sp-Adhi, cauliflower mosaic virus 35S promoter and maize Adh1 intron; CHI, *Manduca sexta* chitinase; UBIp-i, maize ubiquitin promoter and intron; WGA, wheat germ agglutinin.

Transformation. Maize transformation of Hi-II embryos was performed by the Iowa State Plant Transformation Facility using the *A. tumefaciens* protocol.[23] Immature zygotic embryos were excised from kernels of the Hi-II maize line and briefly submerged in liquid cultures of *A. tumifaciens* containing the transformation plasmid. The embryos were cocultivated with the *A. tumifaciens* for 3 days in the dark. After 4−7 days on the resting medium, the embryos were placed on selection medium containing 1.5 mg/L bialaphos. Two weeks later, the embryos were placed on selection medium containing 3 mg/L bialaphos. Putative transgenic callus was multiplied for several weeks before shipping. Transgenic callus was shipped to Peoria, and plants were regenerated according to an established protocol.[23] Callus was initially held in the dark at 25 ± 1 °C. After 1 week, the callus was placed on regeneration medium I for 2−3 weeks. The callus was then grown on regeneration medium II in the light until leaves were at least 2 cm long.

DNA Analysis of Transformants. Genomic DNA was extracted from frozen leaf material using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) or the DNeasy Plant Mini Kit (Quiagen, Valencia, CA, USA), according to the manufacturer's instructions. Primers utilized were 5′-CCTGAAGATCACCCTGTGCT-3′ forward and 5′-CATTAGGTGGTCGGTGAGGT-3′ reverse for actin, 5′-GATATCGCCATGAGAAAGATGATGAGC-3′ forward and 5′-GGAGCTCTCATTCTGCGAGAAGAGT-3′ reverse for the WGA gene, and 5′-GTTGAGTGGCCCTGTTTCTCGG-3′ forward and 5′-CATTGGTGTATGGCGCAGGGTC-3′ reverse for the ADH intron and N-terminal portion of the THWC coding sequence. The 385 bp portion of the maize actin gene was used to determine the quality of extracted genomic DNA prior to testing for the transgenes. Approximately 100 ng of genomic DNA, 15 ng of both forward and reverse primer, 10 μL of PCR Master Mix (Roche Diagnostics, Indianapolis, IN, USA), and 1 μL of DMSO (total mixture volume of 20 μL) were used in polymerase chain reaction (PCR) evaluations. The reaction conditions using a PTC-0150 thermal cycler (MJ Research, Waltham, MA, USA) for the actin amplicon were 94 °C for 2 min; 40 cyles of 94 °C for 30 s, 59 °C for 30 s, 72 °C for 45 s; and 72 °C for 10 min. The same conditions were used for the WGA (ca. 600 bp) and THWC

(ca. 1000 bp) amplicons, except annealing temperatures of 55 and 63 °C were used, respectively.

Protein Analysis of Transformants. Representative samples from 10 different transformation events, showing a range of activity against insects and fungi in 33 different regenerated plants, with at least two plants from every event but one, were analyzed for production of transgenic protein. The amounts of THWC, MRIP, and WGA produced in leaves were quantitated by Western blotting and use of antibodies. MRIP antibody and standard were obtained from R. S. Boston, Department of Botany, North Carolina State University. WGA antibody was purchased from Biogenesis (Kingston, NH, USA), and WGA standard was purchased from Sigma-Aldrich (St. Louis, MO, USA). THWC antibody and standard were obtained from S. Mathukrishnan. Four of the 4 mm diameter (4 mg each) leaf disks used in corresponding bioassays were frozen in liquid nitrogen and ground in 1.5 mL tapered vials using tapered plastic pestles. Extraction buffer, consisting of pH 7.4, 250 mM sucrose, 2 mM EDTA, with complete Ultra Protease Inhibitor (Roche Diagnostics, Indianapolis, IN, USA), was added immediately after grinding, and the tubes were placed on flaked ice to thaw the buffer.

The Western blotting and antibody detection procedure was similar to that reported previously.[12,18] Ground samples in buffer were briefly vortexed and then centrifuged for 15 min at 14000*g* at 4 °C. Then, 26 μL of the supernatant, 10 μL of NuPage 4× LDS sample buffer (Invitrogen), and 4 μL of β-mercaptoethanol were combined and heated for 5 min at 95 °C. NuPage 12% Bis-Tris gels (Invitrogen) were loaded with 20 μL of sample, and the gels were run for 35 min at 200 V. Proteins were transferred to PVDF membrane from the gel in NuPage buffer for 1 h at 30 V. Blots were blocked overnight at 4 °C using a solution of 5% bovine serum albumin fraction V (Sigma-Aldrich) in pH 7.4 phosphate-buffered saline (1.37 M NaCl, 2.6 mM KCl, 101.4 mM Na_2HPO_4, 17.6 mM KH_2PO_4) containing 0.01% Tween 20 (PBST). Primary antibodies were diluted 1:5000 in PBST and incubated for 1 h with shaking. Blots were then washed three times in PBST before the addition of anti-rabbit horseradish peroxidase conjugated secondary antibody (Bethyl Laboratories, Montgomery, TX, USA) diluted 1:5000 in PBST. Blots were again washed three times in PBST. Bands were detected using SuperSignal West Femto (Thermo Scientific, Rockford, IL, USA), and bands were quantitated using a Bio-Rad ChemiDoc XRS+ Imager equipped with Image Lab V 3.01 software (Bio-Rad Laboratories, Richmond, CA. USA). Standards for MRIP were added in the 50 ng−500 pg range, and standards for WGA were added in the 600 ng−60 ng range; molecular weight standards (Page Ruler, Thermo Scientific) ranged from 140 to 10 kDa. Different concentrations for the two proteins were used due to differing sensitivities of the antibodies toward respective proteins, as determined in preliminary experiments. Preliminary investigations indicated the WGA standard (Sigma-Aldrich) reacted more sensitively with the antibody if it was preincubated with buffer containing 0.1% ethylenediamine (EDA) for 1.5 h. Total protein of supernatants was determined using the Bio-Rad protein assay kit using bovine serum albumin as the standard. In a few cases, detectable protein bands fell below the lowest concentration standard, so values were extrapolated from the standard curve. The low concentration protein band that sometimes overlapped with WGA occurred at a concentration that was significantly correlated with protein concentration and so was subtracted as background from WGA values as necessary.

Because there was sometimes overlap with the low concentration native maize protein and minor size differences occurred compared to the standard, WGA presence was confirmed using affinity column purification for representative samples. Leaf pieces weighing from 50 to 100 mg were ground as described above, and buffer was added in a 1:5 ratio. Extracts were centrifuged, and supernatants were applied to a fetuin−agarose column (Sigma-Aldrich) equilibrated with 20 mL of PBS. The applied supernatants were allowed to incubate with columns held horizontally for 1 h at 4 °C with occasional inversion. Columns were washed with 30 mL of PBS and then eluted using 100 mM ethylenediamine; typically most of the WGA eluted in the second 1 mL fraction. Prior to gel application, the supernatant was concentrated to approximately 100 μL using either polysulfone or cellulose 10000*g* cutoff spin columns (Millipore Ultrafree, obtained from Sigma-Aldrich). Concentrates were subjected to Western blotting and detected as described above.

The presence of WGA in the transformed plants was further confirmed using matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS). Samples were prepared by grinding and extracting 50−60 mg of frozen leaf material as described above; both transformed and untransformed tissue were compared. The protein extracts were placed on the fetuin column as before, and three 1 mL fractions were collected. The second and third fractions were combined and dried down in an Eppendorf vacufuge at 60 °C. Protein was resuspended in 20 μL of 100 mM ethylenediamine initially, and then 10 μL of NuPage sample buffer and 4 μL of β-mercaptoethanol were added. Samples were run on a 10% Tricine gel and included 500 ng of WGA standards to help locate the transgenically produced WGA, which would not be visible using the staining procedure needed for MALDI-TOF MS due to the low concentration. The gel was then washed in water for 3 min and then stained with Bio-Rad copper stain for 5 min. The gel was washed again in water for 5 min before slices of the gel were cut out at the appropriate position relative to the WGA standard. Gel slices were placed in 1.5 mL tubes and destained using a Bio-Rad destaining solution and methodology adapted for subsequent MALDI-TOF MS of samples.[24] First, 900 μL of water with 100 μL destain were added and vortexed for 5 min. The solution was then removed and fresh solution added as before. Samples were then vortexed for 10 min, and the solution was removed again. The last solution contained 950 μL of water and 50 μL of destain solution with the samples vortexed for 5 min. The solution was removed, and the gel slice was rinsed with water. Gel slices were dried on absorbant tissue, placed into 0.2 mL tubes, and broken into small pieces with a spatula. Twenty microliters of matrix solution (formic acid/butanol/water (3:5:1 v/v/v) saturated with α-cyano-4-hydroxycinnamic acid) was added, and the samples were then vortexed for 1 h. Samples were kept at 4 °C until cocrystallization onto a standard 96-position stainless steel target. MALDI-TOF MS spectra were recorded on a Bruker-Daltonics Microflex LRF instrument (Bruker-Daltonics, Billerica, MA, USA) running in linear, positive ion mode. Typically, 1000−2000 laser shots were acquired at 60 Hz frequency and 78% laser power, using a pulsed nitrogen laser with the attenuator offset at 16% for 30% range. Ion source 1 was set to 19.0 kV and source 2, to 15.9 kV (83.7% of IS 1), with lens and reflector voltages of 9.79 and 19.99 kV,

Table 1. Range of Biological Activity of Selected Leaves with Relatively Lower and Higher Expression against Insects and Fungi[a]

	expression		corn earworm		fall armyworm		
transformant	MRIP	WGA	feed	wt	feed	wt	*Fusarium verticillioides*
lower expression							
1-6LR	0.61	21.1	98	62	100	192	64
6-1	0.98	19.3	92	94	71	133	85
11-2	0.034	0.00	100	88	76	142	85
11-5	0.0	34.2	83	60	78	117	23
15-2	0.56	37.4	100	44	90	65	60
higher expression							
6-5	2.2	83.3	45	31	53	82	43
9-1	1.1	119	51	42	62	75	10
9-3	1.8	172	55	51	41	70	43
10-6	0.08	138	33	49	22	58	40
14-1	3.3	166	58	73	27	56	10

[a]Expression levels are reported in ng/mg tissue. Bioassay values reported are percentage relative to maximum value for the other leaf material assayed on the same day (feeding and fungal lesion) or relative to weights of larvae fed on artificial diet (weights) to allow for comparisons for assays run on different days (see text).

respectively. During the acquisition matrix ion suppression was used up to 7000 Da. External calibration used Bruker Protein Calibration Standard (Bruker-Daltonics).

Bioassays. Insect bioassays were performed as described previously.[18] Briefly, 2 cm leaf disks were removed from leaves of either seven-leaf or tassel-showing plants, the third leaf below the terminal leaf. Ten caterpillars were added to each leaf disk and held in Petri dishes for 2 days to measure feeding rates (in 0.25 or 1 mm^2 hole equivalents),[18] mortality, and weights of survivors. Portions of the pinto bean diet used for rearing the insects were also used with each set of assays to check and allow for standardization between the different days the assays were run.

Fungal resistance was measured by determining the width of mycelial growth around a cut area where inoculum was introduced. The tip of a watchmaker's screwdriver was sharpened and used to make three equally spaced 2 mm slits in leaf disks. Only leaves from plants showing tassels were used in these assays. Two microliters of fungal spore suspension was added to each slit, and the leaves were placed in Petri dishes containing moistened filter paper as they were for the insect resistance assays. Leaf disks were evaluated for the width of visible mycelial growth under a 20× stereoscope each day for 3 days.

All assay leaf disks that were removed were adjacent to each other on the same leaf. Additional disks of 4 mm diameter were removed from adjacent areas and stored at −80 °C for subsequent quantitation of transgenic protein. Remaining leaf pieces were used for DNA determinations or confirmation of the presence of WGA, MRIP, and THWC using Western blotting, as described above.

Statistical Analysis. Transgenic protein levels were corrected for background and determined on a per milligram total protein basis in Western blotting, using standards. Feeding and fungal lesion values were normalized between different days by adjusting relative to the maximum damage value on the same assay date, which was considered to be 100. Weight values of the 10 caterpillars added to each bioassay material were normalized relative to weights of caterpillars fed on standard artificial diet, which was considered to be 100. Relative contributions of MRIP and WGA to reductions in caterpillar feeding or weights or fungal colonization zone were determined by correlation analysis using SAS PROC REG with option CORR. Potential interactions between the MRIP and WGA in influencing biological responses were determined using SAS PROC REG with MAXR stepwise variable substitution. Outliers were determined using the Diagnostics option of SAS Proc GENMOD, which determines Cook's distance, a useful indicator of potential outliers.[25,26] The SAS programs used were all Windows version 8.0 or higher.

RESULTS AND DISCUSSION

Activity of Transformants against Insects and Fungi. Approximately 60 T_0 transformant plants were examined for effects on insects. Leaves from very few young transformant plants showed obvious feeding differences against the fall armyworm or corn earworm larvae, although some had reductions of near 50% relative to the least severely damaged leaf assayed on the same day. For example, leaf disks from transformant 6-3, which was among the most active transformant plants assayed in older plants, reduced corn earworm feeding by 55% and fall armyworm feeding by 36%. Because CaMV promoters typically express better in more mature tissues, leaves from older transformant plants were additionally examined for activity against insects and fungi. A wide range of activity of the older transformed plants was noted against insects and fungi tested and could be compared for different days of assays after adjustment versus a standard used in each day of the assay, such as weights for insects fed on artificial diet or feeding and fungal colonization on leaves with no transgenic protein expression. Although little mortality was noted against insects (data not shown), significant reductions in feeding and weights of survivors were noted in several cases. Reductions in feeding and growth rates, as reflected by survivor weights, of corn earworms were up to 77 and 72%, respectively, for transformant 6-3. Reductions in feeding and growth rates for fall armyworms were up to 80 and 53%, respectively, for transformant 3-4. Reductions in fungal colonization, as indicated by mycelial growth zone width, were up to 93% for transformant 3-2. More active transformants were generally active against all three species (Table 1), but the maximum effect on a particular assay day for one organism did not necessarily occur for all organisms for a particular transformant.

Detection of Gene Construct in Transformed Plants. The construct was often incorporated in regenerated plants, as approximately 80% of the T_0 transformants examined tested positive by PCR (Figure 2). This rate of incorporation was

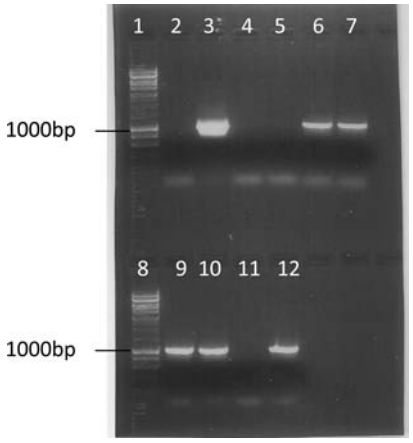

Figure 2. Representative detection of ca. 1000 bp segment including part of the tobacco hornworm chitinase transgene (see text) from genomic DNA of transformants. Lanes: 1 and 8, DNA ladder; 2, no template control; 3, plasmid positive control; 4−12, transformant plants 1-2, 1-3, 3-1, 3-4, 3-5L, 10-2, 11-2, 14-2, respectively. Arrow indicates the expected PCR product.

reflected by the number of transformants in which transformant protein was detected (see below), although a few transformants positive for the gene did not have detectable protein in any of the corresponding plants.

Detection of Target Proteins in Transformed Plants. Despite several attempts, the THWC was never detected. Using a coding triplet frequency table for maize[27] and comparing it to the THWC nucleotide sequence, it was found that several rare coding sequences occurred, especially for cysteine, which may have adversely influenced expression levels. However, both the MRIP (Figure 3) and WGA (Figure 4) were detected in many plants. As expected due to removal of the His-tag and enterokinase site, the MRIP was smaller than the MOD1 standard. Neither MRIP nor WGA was detected in untransformed Hi-II plant leaves (data not shown). Trans-

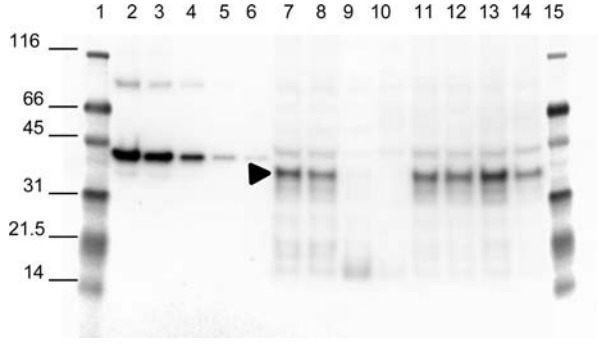

Figure 3. Detection of maize ribosome-inactivating protein (MRIP) in transformants. Lanes: 1 and 15, molecular weight markers; 2−6, MRIP MOD1 standard with concentrations of 50 ng−500 pg; 7−14, transformant plants 3-2, 3-3, 11-1, 11-2, 12-1, 12-2, 14-1, 14-2, respectively. Arrow indicates the expected transgenic protein. The smaller band corresponds to a form with the carboxy-terminal portion removed.[11].

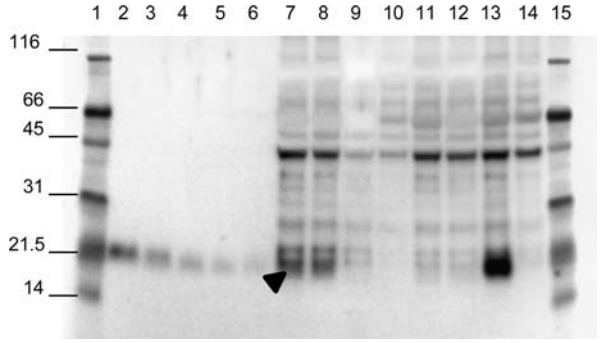

Figure 4. Detection of wheat germ agglutinin (WGA) in transformants. Lanes: 1 and 15, molecular weight markers; 2−6, WGA standard with concentrations of 600 ng−60 ng; 7−14, transformant plants 3-2, 3-3, 11-1, 11-2, 12-1, 12-2, 14-1, 14-2, respectively. Arrow indicates the expected transgenic protein.

formant-produced WGA binding capability was verified by positive detection after affinity purification using a fetuin agarose column (Figure 5), although high levels needed to be

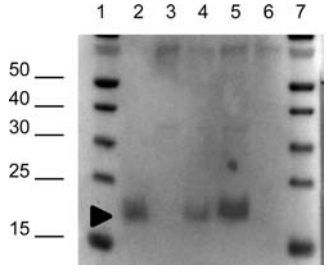

Figure 5. Detection of WGA after affinity column purification. Lanes: 1 and 7, molecular weight markers; 2, WGA standard; 3, wild type column fraction 3; 4−6, transformant 3-5L column fractions 1−3, respectively. Arrow indicates the expected transgenic protein.

present, as indicated by Western blots, to be detectible in column assays. The presence of WGA in the transformed plants was further confirmed by MALDI-TOF MS. A singly charged molecular ion was observed at m/z 18114.3, corresponding to the average mass of the 18 kDa WGA monomer. This ion was also apparent for a commercial standard of wheat germ agglutinin, but was absent from the wild type, negative control (data not shown).

Some transformants had MRIP levels that were in the range of 5 ng/mg (200 ng per mg soluble protein). Some transformants had WGA levels that were in the range of 200 ng/mg (8 μg per mg soluble protein). Plants expressing high levels of one protein did not always express high levels of the other. Of 33 transformants examined for protein levels, after calculated values were corrected for maximum occurrence (i.e., the maximal value was 1.00, and other values were calculated relative to that value), 8 plants had a relative difference of the two proteins >0.5. The rest of the plants had relative levels of MRIP and WGA that were significantly correlated (F = 7.19, P = 0.013, R = 0.49, n = 25). No obvious morphological negative effects were noted on plant growth for any transformants.

Association of Target Protein Levels with Biological Activity of Transformed Plant Leaves. Initial correlation analyses involving all plants indicated only corn earworm feeding, fall armyworm feeding, and fall armyworm weights were significantly negatively correlated with concentrations of WGA (Table 2). WGA, which naturally occurs in mature wheat

Table 2. Correlations of Transformant Protein Levels and Biological Activity

	corn earworm[a]		fall armyworm[a]		
plant *n*	feeding	weight	feeding	weight	*Fusarium verticillioides*
WGA unadjusted					
33	−0.42*	−0.01	−0.44*	−0.44*	−0.13
RIP unadjusted					
33	0.00	−0.09	−0.01	−0.04	0.01
RIP adjusted[b]					
19	−0.45*	0.02	−0.81*	−0.29	−0.08
WGA adjusted[c]					
25	−0.42*	−0.05	−0.38	−0.43*	−0.25
RIP adjusted[c]					
25	−0.46*	−0.01	−0.49*	−0.25	−0.15

[a]Values followed by an asterisk (*) had significant correlations at $P \leq 0.05$. Protein levels were determined on the basis of standards used concurrently on Western blots after correction for background. [b]Adjusted RIP values had all leaf data of 4 events of 10 removed, which had exceptionally low or high RIP values. [c]Leaf values removed for leaves that had relative RIP and WGA values that differed by >0.5.

kernels, has been tested on several insects in diet in prior studies and has activity against caterpillars, beetles, leafhoppers, planthoppers, and aphids.[14] Studies in which WGA has been transgenically expressed in plant tissues and then evaluated for activity against insects have been more limited. When expressed in Indian mustard (*Brassica juncea*), high mortality to the mustard aphid (*Lipaphis erysimi*) was noted in transformants expressing WGA.[28] When expressed in maize callus, reductions in weight gain of European corn borers of up to 95% were found.[14] In regenerated maize plants, only a few had significant activity against European corn borers (*Ostrinia nubilalis*), with up to 60% reduction in feeding and weight reductions up to 50%, with expression levels up to 100 μg/g.[14] Our results indicated WGA could be readily expressed in maize leaves at concentrations that were sufficient to negatively affect feeding and growth of fall armyworms and corn earworms.

Whereas no significant correlations with MRIP concentration and activity against insects or fungi were noted, trends were somewhat similar. Because biological activity needs a specific concentration threshold to be detectible, values below 10 ng/mg soluble protein for RIP transformant plants were removed, which came from plants of two transgenic lines, to investigate if a threshold effect was operating. Graphical plots also indicated four potential outliers at the highest concentrations, which came from plants of two transgenic lines. These were removed, together with one other in the same concentration range. Under these conditions, leaf data from 19 plants of six independent transgenic events indicated activity against corn earworm feeding and fall armyworm feeding, and weights of fall armyworm survivors were significantly negatively correlated with MRIP concentration. MRIP, which accumulates in mature maize kernels, has been examined for activity against insects to a much lesser extent than WGA. When incorporated into diets, MRIP was most active against maize-unadapted insects (cabbage loopers, *Trichoplusia ni*), but much less effective against the Indian meal moth (*Plodia interpunctella*), which feeds on dried maize and other grains.[29] When expressed in tobacco at levels of up to 100 μg/g, MRIP inhibited feeding and reduced growth of both the corn earworm and the cigarette beetle (*Lasioderma serricorne*).[12] In the present study, although a threshold effect was present, MRIP was expressed at sufficient levels in leaves to significantly affect both corn earworms and fall armyworms, in a concentration-dependent manner.

Although trends were noted for fungal activity, no significant correlation was noted between MRIP and WGA or a combination thereof. However, there was some tendency for the size of the mycelial growth zone to be negatively correlated with both MRIP and WGA concentration in some cases. A plot of WGA concentration against *F. verticillioides* growth indicated an effect similar to that noted with MRIP and insect parameters. After removal of high and low values, the concentration of WGA was significantly associated with growth of *F. verticilliodes* ($F = 9.78$, $P = 0.0069$, $R = -0.63$, $N = 17$). A variety of plant proteins have been reported to inhibit plant pathogens, with chitinases being a common component.[10] WGA has been reported to inhibit the growth of two plant pathogenic species of *Fusarium*, including the maize pathogen *F. graminearum*.[15] MRIP inhibited the growth of two species of *Aspergillus*, including one that was a maize pathogen.[30] When expressed transgenically in maize, the degree of resistance to *F. verticillioides* was correlated with the concentration of MRIP detected.[13] It is possible that the concentration of MRIP produced in our transformants was too low to affect the fungi tested, or the strain we used was more resistant to MRIP. We did note some significant correlation of antifungal activity and WGA concentration after outliers were removed.

Potential interactions between the proteins were also examined, using variable addition regression analysis. Under these conditions, only minor interactive effects were noted, suggesting the proteins were mainly acting independently. For example, using the data for 25 transformants where relative differences in RIP and WGA values were <0.50 (Table 2), correlations were increased from −0.46 to −0.51 for corn earworm feeding when WGA values were added to RIP values, from −0.43 to −0.44 for fall armyworm feeding when WGA values were added to RIP values, and from −0.49 to −0.51 for fall armyworm weights, when RIP values were added to WGA values. The activity of MRIP at the concentrations produced in the plants was unexpected, but it is possible the WGA was synergizing the MRIP in some instances. Genetic analyses indicated natural resistance to insects in maize is associated with multiple loci, and the activity of different resistance-associated maize secondary metabolites and proteins has been verified using transgenic studies.[31−33]

Compatibility studies with insect resistance proteins have been limited. Different sources of nonplant chitinase have been coexpressed with a variety of proteins, including the insecticidal *Bacillus thuringiensis* crystal protein and plant proteinases, but no plant-derived chitinases have been combined with other resistance proteins for evaluation.[10] In fact, very few plant-derived resistance proteins have been tested in combination against insects. Potential negative interactions could occur, depending on the proteins used. For example, proteinases may degrade other resistance proteins, and proteinase inhibitors may inhibit defensive proteinases or other hydrolytic enzymes, including chitinases. Plant proteinase inhibitors in different classes acted synergistically against caterpillar and leaf miner pests when expressed transgenically in tomato.[34] When tobacco plants expressing both maize RIP and elevated levels of tobacco anionic peroxidase were evaluated, resistance appeared to be additive, but not synergistic or antagonistic.[35] In the present study, WGA and MRIP were coexpressed in several transformants, and no obvious indication of antagonism (or

synergism) was indicated by statistical analyses. However, the activity of MRIP occurred at a lower level than expected on the basis of prior studies.[12] WGA is reported to produce pores in the peritrophic membrane (the chitinaceous midgut lining that protects absorptive cells) of European corn borers,[16] which could allow for more ready penetration of other bioactive proteins. It is possible that the WGA was opening pores in the peritrophic membrane of the caterpillars examined in the present study and allowing for higher concentrations of MRIP to move to the active site, which is supported by some of our data. In effect, the WGA was synergizing the activity of the MRIP.

Plant proteins have been studied more commonly in combination against fungi than for insects. As for insects, chitinase has been the most commonly investigated protein in combination with other proteins for antifungal activity, but most commonly from nonplant sources. Fungal endo- and exochitinase acted synergistically against the apple pathogen *Venturia inaequalis* when coexpressed transgenically.[36] Chitinase was combined with MRIP in rice, and plants were more resistant to one of three leaf pathogens assayed.[37] Although we noted significant effects on *F. verticillioides* for some transformants, overall activity could be significantly correlated with concentrations of WGA only after outliers were removed.

In conclusion, the present study indicates that MRIP and WGA could be transgenically expressed in maize and increase resistance to both fall armyworms and corn earworms, two major insect pests of maize. Although concentration-dependent effects were noted only for WGA and the maize pathogen *F. verticillioides*, there was a trend for growth inhibition to be negatively correlated with RIP concentration as well. This study verifies the utility of combining genes of different modes of action and that the MRIP and WGA (placeholder for comparable maize lectin) can be components of a defensive strategy against maize pests.

AUTHOR INFORMATION

Corresponding Author

*E-mail: patrick.dowd@ars.usda.gov. Phone: (309) 681-6242. Fax: (309) 681-6593.

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ABBREVIATIONS USED

CaMV, cauliflower mosaic virus; MRIP, maize ribosome-inactivating protein; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline with Tween; PCR, polymerase chain reaction; PVDF, polyvinylidene difluoride; THWC, tobacco hornworm chitinase; WGA, wheat germ agglutinin.

REFERENCES

(1) Pimentel, D. Economic impact of insects. In *Encyclopedia of Plant and Crop Science*; Goodman, R. M., Ed.; Taylor & Francis: Boca Raton, FL, 2004; pp 407−409.

(2) Metcalf, R. L.; Metcalf, C. L. *Destructive and Useful Insects*; McGraw Hill, New York, 1993.

(3) Robens, J.; Cardwell, K. F. The costs of mycotoxin management in the United States. In *Aflatoxin and Food Safety*; Abbas, H. K., Ed.; Taylor & Francis: Boca Raton, FL, 2005; pp 1−12.

(4) Wu, F. Economic impacts of *Fusarium* toxins in animal feeds. *Anim. Feed Sci. Technol.* **2007**, *137*, 363−374.

(5) Liu, F.; Xu, Z.; Zhu, Y. C.; Huang, F.; Wang, Y.; Li, H.; Gao, C.; Zhou, W.; Chen, J. Evidence of field-evolved resistance to CryIAc-expressing Bt cotton in *Helicoverpa armigera* (Lepidoptera: Noctuidae) in northern China. *Pest Manage. Sci.* **2010**, *66*, 155−164.

(6) Storer, N. P.; Babcock, J. M.; Schlenz, M.; Meade, T.; Thompson, G. T.; Bing, J. W.; Huckaba, R. M. Discovery and characterization of field resistance to Bt maize: *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in Puerto Rico. *J. Econ. Entomol.* **2010**, *103*, 1031−1038.

(7) Dhurua, S.; Gujar, G. T. Field-evolved resistance to Bt toxin Cry1Ac in the pink bollworm *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae) from India. *Pest Manage. Sci.* **2011**, DOI: 10.1002/ps2127.

(8) Gassman, A. J.; Petzold-Maxwell, J. L.; Keweshan, R. S.; Dunbar, M. W. Field evolved resistance to Bt maize by western corn rootworm. *PLoS One* **2011**, *6*, e22629.

(9) James, S.; Burton, M. Consumer preferences for GM food and other attributes of the food system. *Aust. J. Agric. Res. Econ.* **2003**, *47*, 501−518.

(10) Dowd, P. F.; Johnson, E. T.; Pinkerton, T. S.; Hughes, S. R. Genetically modified plants containing plant derived genes for broad spectrum insect control to reduce mycotoxins: bioactive proteins. In *Genetically Modified Plants*; Wolf, T., Koch, J., Eds.; Nova Science Publishers: Hauppauge, NY, 2008; pp 127−150.

(11) Krawetz, J.; Boston, R. S. Substrate specificity of a maize ribosome inactivating protein differs across diverse taxa. *Eur. J. Biochem.* **2000**, *267*, 1966−1974.

(12) Dowd, P. F.; Zuo, W. Z.; Gillikin, J. W.; Johnson, E. T.; Boston, R. S. Increased resistance to *Helicoverpa zea* in tobacco expressing an activated form of maize ribosome inactivating protein. *J. Agric. Food Chem.* **2003**, *51*, 3568−3574.

(13) Lanzanova, C.; Giuffrida, M. G.; Motto, M.; Baro, C.; Donn, G.; Hartings, H.; Lupotto, E.; Careri, M.; Elviri, L.; Balconi, C. The *Zea mays* L. b-32 ribosome inactivating protein efficiently inhibits growth of *Fusarium verticillioides* on leaf pieces *in vitro*. *Eur. J. Plant Pathol.* **2009**, *124*, 471−482.

(14) Czapla, T. H. Plant lectins as insect control proteins in transgenic plants. In *Advances in Insect Control: The Role of Transgenic Plants*; Carozzi, N., Koziel, M., Eds.; Taylor & Francis: New York, 1997; pp 123−138.

(15) Ciopraga, J.; Gozia, O.; Tudor, R.; Brezuica, L.; Doyle, R. J. *Fusarium* sp. growth inhibition by wheat germ agglutinin. *Biochim. Biophys. Acta* **1999**, *1428*, 424−432.

(16) Cavalieri, A.; Czapla, T.; Howard, J.; Rao, G. Larvicidal lectins and plant insect resistance based thereon. U.S. Patent 5,407,454, 1995.

(17) Dowd, P. F. Toxicological and biochemical interactions of the fungal metabolites fusaric acid and kojic acid with xenobiotics in *Heliothis zea* (F.) and *Spodoptera frugiperda* (J.E. Smith). *Pestic. Biochem. Physiol.* **1988**, *32*, 123−134.

(18) Dowd, P. F.; Johnson, E. T.; Pinkerton, T. S. Oral toxicity of *β*-*N*-acetyl hexosaminidase to insects. *J. Agric. Food Chem.* **2007**, *55*, 3421−3428.

(19) Lukaszewicz, M.; Feurmann, M.; Jérouville, B.; Stas, A.; Boutry, M. *In vivo* evaluation of the context sequence of the translation initiation codon in plants. *Plant Sci.* **2000**, *154*, 89−98.

(20) Sambrook, J.; Russell, D. W. *Molecular Cloning*; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 2001.
(21) Sheen, J. Protein phosphatase activity is required for light-inducible gene expression in maize. *EMBO J.* **1993**, *12*, 3497−3505.
(22) Wakasa, Y.; Yasuda, H.; Takaiwa, F. High accumulation of bioactive peptide in transgenic rice seeds by expression of introduced multiple genes. *Plant Biotechnol. J.* **2006**, *4*, 499−510.
(23) Frame, B. R.; Shou, H.; Chikwamba, R. K.; Zhang, Z.; Xiang, C.; Fonger, T.; Pegg, E. E. K.; Li, B.; Nettleton, D. S.; Pei, D.; Wang, K. *Agrobacterium tumefaciens* mediated transformation of maize embryos using a standard binary vector system. *Plant Physiol.* **2002**, *129*, 13−22.
(24) Cohen, S. L.; Chait, B. T. Mass spectrometry of whole proteins eluted from sodium dodecyl sulfate-polyacrylamide gel electrophoresis in gels. *Anal. Biochem.* **1997**, *247*, 257−267.
(25) Montgomery, D. C.; Peck, E. A.; Vining, G. G. *Introduction to Linear Regression Analysis*, 3rd ed.. Wiley, NY, 2003.
(26) Walfish, S. A review of statistical outlier methods. *Pharm. Technol.* **2006**; www.pharmtech.com/pharmtech/content/printContentPopup.jsp?id=384716 (accessed Oct 3, 2012).
(27) Kazusa DNA Research Institute. Codon Usage Database, Maize. 2012; www.kazusa.or.jp/codon/cgibin/showcodon.cgi?species=4577 (accessed Oct 4, 2012).
(28) Kanrar, S.; Venkateswari, J.; Kirti, P. B.; Chopra, V. L. Transgenic Indian mustard (*Brassica juncea*) with resistance to the mustard aphid *Lipaphis erysimi*. *Plant Cell Rep.* **2002**, *20*, 976−981.
(29) Dowd, P. F.; Mehta, A. A. D.; Boston, R. S. Relative toxicity of the maize ribosome inactivating protein to insects. *J. Agric. Food Chem.* **1998**, *46*, 3775−3779.
(30) Nielsen, K.; Payne, G. A.; Boston, R. S. Maize ribosome-inactivating protein inhibits normal development of *Aspergillus nidulans* and *Aspergillus flavus*. *Mol. Plant−Microbe Interact.* **2001**, *14*, 164−172.
(31) Pechan, T.; Ye, L.; Chang, Y.-M.; Mitra, A.; Lin, L.; Davis, F. M.; Williams, W. P.; Luthe, D. S. A unique 33-kd cysteine protease accumulates in response to larval feeding in maize genotype resistant to fall armyworm and other *Lepidoptera*. *Plant Cell* **2000**, *12*, 1031−1040.
(32) Johnson, E. T.; Berhow, M. A.; Dowd, P. F. Expression of a maize *Myb1* transcription factor driven by a putative silk-specific promoter significantly enhances resistance to *Helicoverpa zea* in transgenic maize. *J. Agric. Food Chem.* **2007**, *55*, 2998−3003.
(33) Dowd, P. F.; Johnson, E. T.; Pinkerton, T. S. Identification and properties of insect resistance-associated maize anionic peroxidases. *Phytochemistry* **2010**, *71*, 1289−1297.
(34) Abdeen, A.; Virgós, A.; Olivella, E.; Villanueva, J.; Avilés, A.; Gabarra, R.; Prat, S. Multiple insect resistance in transgenic tomato plants over-expressing two families of plant proteinase inhibitors. *Plant Mol. Biol.* **2005**, *57*, 189−202.
(35) Dowd, P. F.; Holmes, R. A.; Pinkerton, T. S.; Johnson, E. T.; Lagrimini, L. M.; Boston, R. S. Relative activity of a tobacco hybrid expressing high levels of a tobacco anionic peroxidase and maize ribosome-inactivating protein against *Helicoverpa zea* and *Lasioderma serricorne*. *J. Agric. Food Chem.* **2006**, *54*, 2628−2634.
(36) Bolar, J. P.; Norelli, J. L.; Harman, G. E.; Brown, S. K.; Aldwinckle, H. S. Synergistic activity of endochitinase and exochitinase from *Trichoderma atroviride* (*T. harzianum*) against the pathogenic fungus *Venturia inaequalis* in transgenic apple plants. *Transgenic Res.* **2001**, *10*, 533−543.
(37) Kim, J. K.; Jang, I. C.; Wu, R.; Zuo, W. N.; Boston, R. S.; Lee, Y. H.; Ahn, I. P.; Nahm, B. H. Co-expression of a modified maize ribosome-inactivating protein and a rice basic chitinase gene in transgenic rice plants confers enhanced resistance to sheath blight. *Transgenic Res.* **2003**, *12*, 475−484.