Enhanced Resistance to *Helicoverpa zea* in Tobacco Expressing an Activated Form of Maize Ribosome-Inactivating Protein

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Progeny of two transgenic tobacco (*Nicotiana tabacum* L.) lines that expressed an activated form of maize (*Zea mays* L.) ribosome-inactivating protein (RIP) had varying resistance to the insect species tested. A subset of R_2 plants from the two lines appeared to be more resistant to larvae of the cigarette beetle, *Lasioderma serricorne* (F.), and the tobacco hornworm, *Manduca sexta* (L.) than the wild type plants. Progeny (R_3) of the more resistant R_2 plants were tested more extensively for insect resistance. Resistance to the corn earworm, *Helicoverpa zea* (Boddie), was most consistent, with significantly decreased feeding often accompanied by increased mortality and reduced weights of survivors fed on leaf disks of the two transgenic lines compared to the wild type. The amount of damage by *H. zea* was significantly inversely correlated with levels of RIP. Resistance of RIP-producing plants to *H. zea* was greater than expected on the basis of prior in vitro results using diet-incorporated maize RIP. The R_3 transgenic plant leaf disks were also often more resistant to feeding by larvae of *L. serricorne* compared to wild type plants. Although reduced feeding by *M. sexta* was noted when they were fed leaf disks from transgenic compared to wild type plants the first day of exposure, differences were not significant. This information provides further support for maize RIP having a role in resistance to maize-feeding insects.

KEYWORDS: Corn earworm; tobacco hornworm; *Manduca*; cigarette beetle; *Lasioderma*

INTRODUCTION

Insects not only cause losses through physical damage but can also spread plant pathogens that produce toxins harmful to people and animals, such as mycotoxins (*1*). Plant resistance can be a useful strategy for insect pest management, but conventional breeding to introduce resistant traits can be a long and labor-intensive process. Biotechnology has provided a new strategy for incorporating insect resistance proteins into plants. Individual genes coding for plant-derived enzyme inhibitors or lectins have been successfully introduced into nonsource plants to produce transgenic plants with enhanced resistance to insects (*2*). Because of concern for resistance development in insects feeding on transgenic plants expressing high levels of a single protein (e.g., ref *3*), additional proteins that have activity against insects are of interest so that complexes can be used to produce multigenic resistance. This type of resistance is theoretically more stable toward resistance development by insects (*3*), much like the multigenic resistance to different pests that appears to occur naturally in plants (e.g., ref *4*).

Plant-derived ribosome-inactivating proteins (RIPs) are *N*-glycosidases that remove the purine ring from a specific adenine of ribosomal RNA, resulting in inhibition of protein synthesis (*5*). Plant RIPs are active against some insects, although the spectrum of activity varies according to the RIP source plant (*6*, *7*). Plant-derived RIPs are typically inactive against Lepidoptera at concentrations that are toxic to Coleoptera (*6*, *7*), but the maize RIP is an exception (*7*). Maize RIP is proteolytically activated by removal of amino acids at the two ends and in a central region, with the major polypeptide products associating to form the active molecule (*8*, *9*). Previously we reported that the proteolytically activated maize RIP is active at naturally occurring concentrations against most maize-feeding and other caterpillars tested (*7*). Here we report on the enhanced resistance of transgenic tobacco, *Nicotiana tabacum* L., that produces activated maize RIP to feeding by *Helicoverpa zea* (Boddie), a major caterpillar pest on tobacco and many other crops, and by *Lasioderma serricorne* (F.), a major stored-product beetle pest.

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MATERIALS AND METHODS

Insects. False tobacco budworms (corn earworm, *H. zea*) were reared on a pinto bean based diet at 27 ± 1 °C, 14:10 light/dark photoperiod, and $40 \pm 10\%$ relative humidity as described previously (*10*). Eggs of tobacco hornworms, *Manduca sexta* (L.), were obtained from Carolina Biological Supply (Burlington, NC) and were hatched under the same conditions used to rear the *H. zea*. Newly hatched, first instar caterpillar larvae were used in bioassays. Cigarette beetles (*L. serricorne*) were reared on corn meal, flour, and brewer's yeast at 27 ± 1 °C, 14:10 light/dark photoperiod, and $60 \pm 10\%$ relative humidity as described previously (*11*). Second-instar *L. serricorne* larvae were used in bioassays. Adult greenhouse whiteflies [*Trialeurodes vaporariorum* (Westwood)] were obtained from infested greenhouse plants.

Construction of Transgenes and Plant Transformation. An active maize RIP construct (MOD1) has been described previously (*12*). The active RIP coding sequence was excised from MOD1 and inserted between a 35S CaMV promoter and NOS terminator in a modified pBIN19 vector (*13*). In this vector, translation of the active RIP would be initiated at the first ATG within the coding sequence to produce a 26 kDa protein product lacking the 35 N-terminal amino acids and the 27 internal amino acids of the proenzyme (*9*). The RIP construct was introduced into *Agrobacterium tumefaciens* strain LBA4404 by transformation. Gene introduction into *N. tabacum* cv. KY14 leaf disks was carried out according to the method of Horsch et al. (*14*). Plant tissues used for insect bioassays in the present study were grown from R_1 and R_2 seed of two independent RIP transformant lines (5-1 and 5-7) germinated on kanamycin as described below. Leaves were removed from different generations of plants and assayed for maize RIP by quantitative immunoblot analysis (see below).

RIP Gene Characterization and Protein Quantitation in Transformants. Initial transformants that had good seed set and no visible phenotypic differences (plants 5-1 and 5-7) were assayed for the presence and expression of the transgene using Southern blots and northern blots. For Southern analysis, genomic DNA was isolated from tobacco leaves according to the CTAB method (*15*) except that RNase treatment was performed during restriction enzyme digestion. Ten micrograms of genomic DNA was digested with *Hin*dIII, separated through 0.7% agarose gels and transferred to Hybond-N+ membranes (Amersham, Piscataway, NJ). Hybridization was carried out according to the method of Church and Gilbert (*16*) with a probe from the coding region of the proRIP gene radiolabeled with [^{32}P]dCTP by random priming. R_1 progeny of transformants 5-1 and 5-7 were checked for the presence of the construct using PCR analysis, using primers 5′-atgacgcacaatcccactat-3′ (within the CaMV promoter) and 5′-ggcctc-gagcatcttcttcttcttcgcc-3′ (within the maize RIP gene).

The quantity of RIP in the soluble protein fraction from leaves was determined with anti-maize proRIP antibody (*9*). Initial R_0 plants, as well as a subset of plant tissue from older R_3 plant leaves used in insect bioassays, were examined using SDS denaturation and RIP quantitation. Extraction buffer [0.1 M NaPO$_4$, pH 7.4, 0.33% (w/v) DTT, and 0.33% (w/v) PVP] (individual components, Sigma Chemical Co., St. Louis, MO) was added 9:1 (v/w) to the leaf tissue and homogenized in a ground glass dounce homogenizer. Samples equivalent to 3 mg of tissue were clarified by centrifugation at 16000g for 10 min. The R_0 leaf homogenates were also subfractionated with (NH$_4$)$_2$SO$_4$, and the 50−75% subfraction was used for analysis. Clarified samples were adjusted to 1× SDS-PAGE loading buffer and fractionated through 12% SDS-PAGs as described previously (*9*). Proteins were transferred onto Immobilon PVDF membranes (Millipore Corp., Burlington, MA) using a submersible system with 48 mM Tris/39 mM glycine buffer (pH 9.2).

Membranes were probed overnight with anti-maize proRIP antibodies (1:10000 dilution) in 0.01 M Tris, 0.8% (w/v) NaCl, and 0.1% (v/v) Tween-20 (TST) after blocking with TST and 5% (w/v) evaporated milk (Carnation, Solon, OH). Primary antibodies were detected with HRP-conjugated goat anti-rabbit IgG (Bio-Rad, Richmond, CA) at 1:10000 dilution. Immunoblots were developed with the SuperSignal West Pico solution (Pierce, Rockford, IL). Different concentrations of MOD1, a recombinant maize RIP construct expressed in *Escherichia coli* (*12*), were used as a standard for the SDS-PAGE separations. Recombinant MOD1 purified from *E. coli* contains additional N-terminal amino acids that result in a polypeptide $\sim$4200 kDa larger than the polypeptide produced in transgenic plants.

To determine RIP levels in transformant progeny leaves used in R_3 bioassays, 10 mg leaf disks taken at the same time from the same leaves used in bioassays were homogenized in 100 μL of pH 7.4, 0.1 M sodium phosphate buffer containing 0.5% DTT and 0.5% PVP using a small-volume ground glass homogenizer (no. 612, Radnoti Glass Technology, Arcadia, CA). The homogenate was centrifuged at 10000g for 15 min, and the supernatant was used for assays. Due to interest in the electrophoretic behavior of the transgenic RIP protein in its native form, the proteins were fractionated on 7.5% polyacrylamide gels under non-denaturing conditions in the cathodic direction for 1 h as described previously (*17*). Proteins were transferred onto Immobilon PVDF membranes using a Novablot semidry transfer system (Amersham-Pharmacia Biotech, Piscataway, NJ) at 160 mA for 1 h in a pH 8.6, 0.1 M Tris glycine buffer containing 10% methanol set up in a continuous system. RIP present on the membranes was visualized using anti-maize proRIP antibody as just described. Maize-derived activated RIP standards produced from prior work (*7*) run concurrently on gels were used for quantitation purposes. In addition to use of standards of different concentration, different exposure times were used to further assist in the visual estimation of quantities of RIP present.

Plant Growth Conditions. Wild type and transgenic *N. tabacum* plants used for insect bioassays were grown at 27 ± 1 °C, $50 \pm 10\%$ relative humidity, and 14:10 light/dark photoperiod in growth chambers (EGC model G-10, Chagrin Falls, OH) as described previously for other tobacco varieties (*18*). Seed produced by 5-1 and 5-7 R_0 transformant progeny (R_1 plants) was sterilized in 10% bleach (5% sodium hypochlorite) containing a few drops of 10% (w/v) sodium dodecyl sulfate (SDS) (Sigma Chemical Co.) for 20 min and then rinsed three times in sterile water. Seed was selected for positive transformants by placement on Murashige and Skoog basal salts with minimal organics (MSMO) medium (Sigma Chemical Co.) containing 0.01% kanamycin in growth chambers under conditions as just described. Seedlings that survived for 1 week on the selective medium were transferred to soil mix (Ready Earth, Scotts-Sierra Horticultural Products, Marysville, OH; *18*) and grown in the growth chamber under described conditions. Bioassay of these plants indicated only a few appeared to be more resistant to feeding by *M. sexta* and *L. serricorne* compared to wild type plants (see below) (*H. zea* was not tested). Seed from the three most active plants of each of the two transformant lines was germinated in groups in soil and individually transplanted when seedlings reached 1 week of age for a second series of more extensive bioassays (see below). Wild type seed was similarly handled and tested during each series of bioassays with the transgenic plants so that similarly aged seed from wild type plants was available for each series of bioassays. Once bioassays were performed, plants were moved to a greenhouse or growth room for seed production at 18 ± 2 °C night (10 h), 29 ± 2 °C (day), $50 \pm 10\%$ relative humidity, and 14:10 light/dark temperature and photoperiod.

The most recently mature leaves were removed for bioassays once leaves reached $\sim$6 cm in length [$\sim$10−14 days after transfer to soil (R_2) or planting (R_3)]. Recently mature leaves from the same plants were used again $\sim$2 weeks later. Disks were cut from the leaves using cork borers. A 20 mm diameter disk was removed from each leaf for bioassays, and a 5 mm diameter disk was removed from the same leaf at the same time (see below) and stored at −20 °C for subsequent RIP quantitation. Leaf disks were removed from the same portion of each leaf for each series of bioassays, with the smaller disk removed closer to the leaf tip. Not all leaf types were used with all insects for different sets of assays; specifics are indicated in the tables.

Bioassays. Bioassays with *H. zea*, *M. sexta*, and *L. serricorne* were performed as described previously (*18−20*). Leaf disks were placed individually in 5 cm diameter Petri dishes with tight-fitting lids (Falcon 1006, Becton Dickinson, Lincoln Park, NJ). The number of insects added to the leaf disk depended on the size of the insect. Either 10 newly hatched *H. zea*, 2 newly hatched *M. sexta*, or 5 second-instar ($\sim$1 mg) *L. serricorne* larvae were added. Plates were held in the dark under the same conditions used to rear the insects. Leaf disks were rated for cumulative feeding damage after 1, 2 (caterpillars) 4, or 6−7
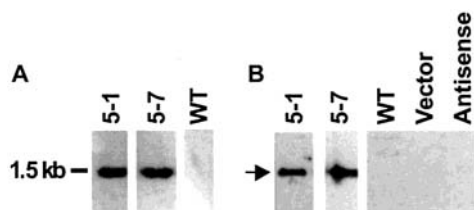
Figure 1. (A) Southern blot of *Hin*dIII cleaved tobacco genomic DNA from R_0 maize RIP transformant plants. Samples from the two independent transformants 5-1 and 5-7 are shown in the left two lanes. These plants were the progenitors of plants used in the present insect resistance work. The position of the predicted 1.5 kb fragment is marked. DNA from an untransformed tobacco line (WT) is show as a control. (B) Immunoblot analysis of a 50–75% $(NH_4)_2SO_4$ protein fraction from leaves of R_0 maize RIP transformant plants. Proteins were separated by SDS-PAGE through 12% gels, transferred to membranes, and probed with antibody against maize proRIP. The arrow shows the RIP signal. The two left lanes contain protein from the 5-1 and 5-7 transformants. The right three lanes contain protein from an untransformed tobacco line (WT), a line transformed with vector alone (Vector), and a line transformed with the RIP clone placed in the antisense direction relative to the promoter (Antisense).

(*L. serricorne* only) days. Feeding damage was rated by counting the number of 0.25 mm^2 (*H. zea* only, approximately larval head-capsule size) or 1 mm^2 (*L. serricorne* and *M. sexta*) holes and estimating equivalent area damage when holes were confluent (*18−20*). In cases when most leaf disk damage exceeded $^1/_{10}$ of the total leaf disk area for any species, damage was recorded using a 1−10 scale based on tenths of leaf damaged (*20*). Mortality was determined after 2 days (or 5−7 days for *L. serricorne*), and survivors were weighed using an analytical balance. Because there was some minor variation in growth rates for both transgenic and wild type plants, separate batches of assays were sometimes run on different days. However, at least some wild type plant leaf disks were paired with at least some transgenic leaf disks in all assays. Choice assays were also performed with *H. zea* and *M. sexta*, using one leaf disk of each type from the same leaf used for no-choice assays, but with twice the number of caterpillars as described previously (*20*). Bioassays with adult *T. vaporariorum* (∼1 week old) were run on ∼2-month-old R_2 plants using 3 cm diameter clip-on cages, which were placed on the leaves that had most recently reached full size (*20*). Numbers of insects and plants used were dependent on insect availability; however, there was always sufficient leaf tissue for ad libitum feeding. Numbers of adults used per cage ranged from 3 to 16. Both control and transformed plants were used in batches of assays set up on a particular date. Replications for assays are indicated under Results.

Statistical Analyses. Statistical analyses were run using SAS software (*21*). Mortality data were analyzed with chi square analysis using PROC FREQ, and the log likelihood ratio statistic was used when cell values were <5. Weight and feeding rating data were analyzed using ANOVA with PROC GLM or equivalent. Correlations between feeding damage and RIP levels were run using PROC REG option CORR. To standardize data for correlation analyses between different date assays using the same insect species and plant type, the individual 2-day feeding rating was converted to a ratio relative to the mean wild type leaf feeding value for that particular series of assays.

RESULTS

RIP Gene Characterization and Protein Quantitation. The southern blot of the R_0 leaf material indicated the presence of a 1.5 kb fragment that cross-hybridized to the probe, indicating that no major rearrangements or deletions occurred during transformation (**Figure 1A**). The northern blot analysis also indicated the presence of RNA of the inserted gene (data not shown). PCR analysis of R_1 plants yielded a product of expected size, indicating the presence of the gene construct in subsequent
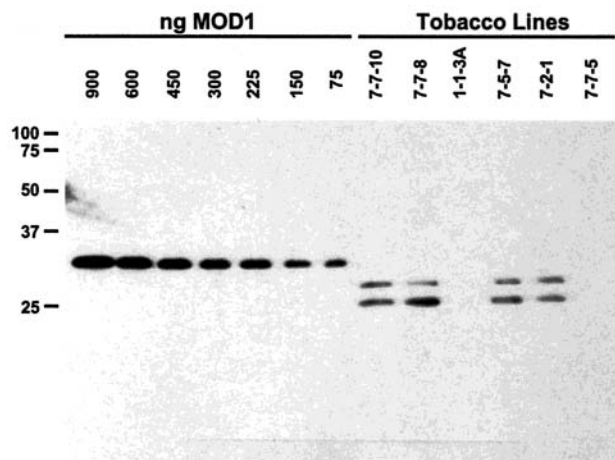

Figure 2. Presence of maize RIP in transgenic tobacco plants when separated by denaturing SDS gel electrophoresis and detected with RIP antibody, showing different concentrations of RIP standards. Four moderate to strong positive 5-7 transformants (7-7-10, 7-7-8, 7-5-7, and 7-2-1) are shown. Molecular weight marker designations in kilodaltons are indicated on the left side. Although not shown on these examples, no wild type plants tested yielded any positive reactions.

generations of transformants that expressed the RIP protein (see below) but not in wild type plants (data not shown).

The R_0 plants produced the expected RIP protein, as indicated by the western blots probed with anti-maize proRIP antibody (**Figure 1B**). A single cross-reacting band was detected in the 5-1 and 5-7 lines used in subsequent experiments. No corresponding cross-reacting bands were detected in wild type tobacco, or a transgenic tobacco harboring an empty vector, or a construct in which the RIP gene was inserted in the antisense orientation (**Figure 1B**). Stable incorporation of the transgene was confirmed by kanamycin germination of seed and RIP protein production in subsequent generations of plants derived from lines 5-1 and 5-7.

The maize RIP produced in the transgenic tobacco plants appeared to be an intact protein with expected migration somewhat faster than the MOD1 standard on denaturing gels (**Figure 2**) and slower than the activated maize RIP-derived standard on native gels (**Figure 3**), which was as expected in both cases. On denaturing gels, an additional, slightly smaller band was seen at a position expected if the carboxy terminal portion had been removed (**Figure 2**). Extracts of wild type plants did not yield positive signals. Calculated mean values of maize RIP for all transformants examined in native gels were 34.7 ± 7.4 ppm for the 5-1 series and 78.3 ± 7.9 ppm for the 5-7 series (means $\pm$ standard errors for 30 of the 5-1 plants and for 26 of the 5-7 plants). At least some of the R_3 plants derived from each of the R_2 parents had levels of maize RIP approaching 100 ppm in leaves.

R_1 Progeny (R_2) Assays. Significantly lower feeding rates by *M. sexta* on transgenic versus wild type leaf disks were noted only for the 5-1 transformants on day 1 (**Table 1**). There were no significant ($P > 0.05$) overall differences in mortality or weights between the *M. sexta* larvae fed on transgenic versus wild type leaf disks. There were no significant ($P > 0.05$) overall differences in feeding rates for *L. serricorne* larvae except for larvae that fed on transgenic leaf disks from the 5-7 transgenic line compared to the wild type leaf disks by day 2. There were no significant ($P > 0.05$) overall differences in mortality or survivor weights for *L. serricorne* larvae fed on wild type versus transgenic leaf disks from either transgenic line. The mortality
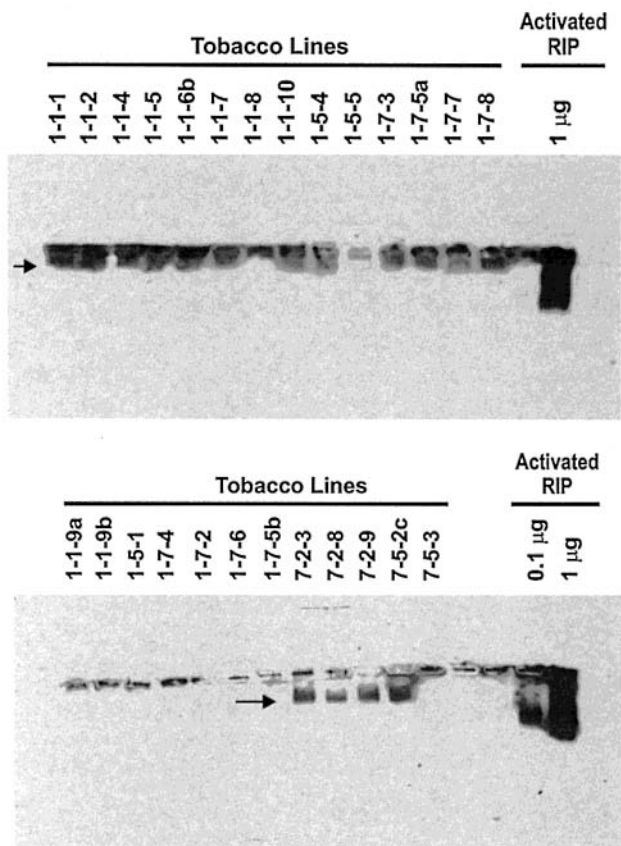
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Figure 3. Presence of maize RIP in R_3 transgenic tobacco plants when separated by native polyacrylamide gel electrophoresis and detected by RIP antibody. Arrows indicate position of the RIP transformant bands. (A) 5-1 transformants, showing some moderate (1-1-1, 1-1-2, 1-1-4, 1-7-5a, and 1-7-8) and weak (1-1-5, 1-1-6b, 1-1-7, 1-1-10, 1-5-4, 1-7-3, and 1-7-7) positives. (B) 5-7 transformants, showing four strong positives (7-2-3, 7-2-8, 7-2-9, and 7-5-2c). Standards are shown on the far right lane-(s) and are activated native maize RIP, which runs farther due to its smaller size (slight charge differences may also occur). Gel for **Figure 1A** was run for a shorter period of time than for **Figure 1B**, hence the shorter migration rates of the bands. Although not shown on these examples, no wild type plants tested yielded any positive reactions. Not all standards used for quantitation are shown on these examples.

of *T. vaporariorum* caged on transgenic line 5-1 (87.3% for 89 total insects caged on 9 different plants) was significantly greater (*P* < 0.05) than mortality of *T. vaporariorum* caged on transgenic line 5-7 (51.5% mortality for 34 total insects caged on 8 plants) or wild type plants (53.9% mortality for 48 total insects caged on 13 plants), which were not significantly different from each other.

After examination of individual effects of the different transgenic plants on insects in the different assays, however, it appeared that some of the plants had much greater activity against the insects than others of the same transformant line. For the 5-7 line, three R_2 plant leaf disks had significantly less feeding by *L. serricorne* at day 6 than the others (18.0 ± 1.4 versus 31.8 ± 1.8 mm², respectively, *P* = 0.012). The same relationship was seen for *M. sexta* that fed on leaf disks from the same plants (4.3 ± 0.7 versus 7.3 ± 0.5 tenths of leaf disk, respectively, *P* = 0.012). This type of relationship was less clear for R_2 plants of the 5-1 transformant line, but again disks from three plants tended to have lower feeding rates than the other transformants, although differences between feeding rates from these plants versus others were not significant (for *M. sexta*,

Table 1. Effect of Wild Type and Transgenic Maize RIP Tobacco R_2 Plant Leaf Disks on *M. sexta* and *L. serricorne*[a]

		feeding rate[b]		mortality	survivor	plant
series		day 1	day 2	(%)	wt (mg)	N
M. sexta						
5-1	WT	2.7 ± 0.3a	8.4 ± 0.4a	0.0a	7.6 ± 0.3a	10
	TG	1.4 ± 0.2b	6.6 ± 0.9a	0.0a	6.3 ± 0.8a	7
5-7	WT	23.3 ± 1.4a	4.1 ± 0.3a	0.0a	ND	20
	TG	20.4 ± 3.1a	6.4 ± 0.6a	0.0a	ND	10
L. serricorne						
5-1	WT	0.8 ± 0.1a	1.5 ± 0.2a	6.3a	2.0 ± 0.1a	10
	TG	0.6 ± 0.1a	1.3 ± 0.3a	0.0a	1.7 ± 0.1a	7
5-7	WT	9.2 ± 1.0a	20.6 ± 1.4a	15.2a	1.1 ± 0.1a	20
	TG	11.3 ± 1.5a	26.3 ± 2.6b	6.4a	1.1 ± 0.1a	10

[a] Mortality values based on a 2 day interval for *M. sexta* and a 5 day interval for *L. serricorne*. Feeding rate and weight values are means ± standard errors and are cumulative over time. Values in columns for like studies followed by different letters are statistically different at *P* < 0.05 by chi square (mortality) or ANOVA (feeding rates and survivor weights). WT, wild type; TG, transgenic; ND; not determined. [b] Feeding rate values are in mm² for *L. serricorne* 5-7 leaf disks and day 1 of *M. sexta* 5-7 leaf disks; the rest of the values are in 1/10s of leaf consumed.

Table 2. Effect of Wild Type and Transgenic Maize RIP Young Tobacco R_3 Plant Leaf Disks on *H. zea*[a]

		feeding rate (0.25 mm²)		% mortality		survivor	
plant series		day 1	day 2	day 1	day 2	wt (mg)	N
5-1							
set 1	WT	42.9 ± 5.4a	173.0 ± 24.3a	4.3a	1.4a	0.14 ± 0.01a	9
	TG	16.5 ± 1.4b	43.7± 5.8b	20.6b	10.4a	0.05 ± 0.01b	12
set 2	WT	27.0 ± 2.0a	74.7± 7.2a	5.1a	7.2a	0.08 ± 0.01a	16
	TG	17.3 ± 3.1b	39.6± 7.0b	19.1b	15.2a	0.07 ± 0.01a	14
set 3	WT	23.8 ± 3.1a	94.5 ± 17.4a	15.9a	12.2a	0.08 ± 0.01a	6
	TG	11.3 ± 1.5b	36.5± 4.8b	14.0a	7.7a	0.04 ± 0.01b	6
5-7							
set 1	WT	31.4 ± 2.9a	99.4± 3.9a	9.2a	13.6a	0.14 ± 0.01a	11
TG	TG	20.3 ± 2.4b	77.0± 6.6a	19.7a	12.3a	0.13 ± 0.01a	9
set 2	WT	26.8 ± 4.0a	93.0 ± 12.4a	14.1a	15.1a	0.07 ± 0.01a	10
	TG	12.8 ± 2.3b	42.6± 8.8b	32.2b	19.0a	0.05 ± 0.01b	11
set 3	WT	64.7 ± 1.9a	157.7± 3.5a	0.0a	4.3a	0.13 ± 0.02a	3
	TG	46.8 ± 6.2b	95.2 ± 16.4b	2.0a	5.6a	0.11 ± 0.02a	6

[a] Feeding rate and weight values are means ± standard errors and are cumulative over time. Values in columns for like studies followed by different letters are statistically different at *P* < 0.05 by chi square (mortality) or ANOVA (feeding rates and survivor weights). Mortality values are based on found larvae. Due to cannibalism of dead larvae, day 2 mortality values may be lower than day 1 values. WT, wild type; TG, transgenic.

feeding rates were 5.0 ± 1.0 versus 7.8 ± 1.1 tenths of leaf disk, respectively, with *P* = 0.121). We selected seed from the group of more resistant transgenic plants of each series for further bioassay (R_3 series).

Selected R_2 Progeny Assays (R_3). The amount of feeding by *H. zea* larvae on leaf disks from younger R_3 plants of both transgenic lines was significantly less than for corresponding wild type disks in all but one case (**Table 2**). The mortality rate of larvae fed the transgenic leaf disks was significantly greater than that of those fed wild type leaf disks in some of the sets after 1 day, but not after 2 days (**Table 2**). There was a general trend for weights of surviving larvae fed R_3 transgenic leaf disks to be significantly less than those fed wild-type leaf disks, which were significant at *P* < 0.05 in half of the studies (**Table 2**). Feeding by *H. zea* on leaf disks from older R_3 plants generally followed the same trend as for feeding on younger plant leaf disks, with frequent significant reductions in feeding on transgenic compared to wild type disks (**Table 3**). The

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Table 3. Effect of Wild Type and Transgenic Maize RIP Older Tobacco R_3 Plants on *H. zea*[a]

plant series		feeding rate (0.25 mm²)		% mortality		survivor wt (mg)	N
		day 1	day 2	day 1	day 2		
5-1	WT	21.7 ± 2.2a	40.8 ± 4.9a	24.1a	25.8b	0.07 ± 0.01a	15
	TG	14.4 ± 1.8b	34.8 ± 3.7a	35.1a	18.1a	0.05 ± 0.01b	15
5-1	WT	28.2 ± 3.1a	54.2 ± 5.6a	23.9a	38.6a	0.05 ± 0.01a	9
	TG	18.9 ± 3.2b	28.5 ± 4.7b	40.3b	55.4a	0.03 ± 0.01b	10
5-7	WT	19.5 ± 1.8a	39.4 ± 3.4a	23.2a	27.5a	0.07 ± 0.01a	13
	TG	9.0 ± 0.7b	15.6 ± 0.9b	42.9b	40.3a	0.04 ± 0.01b	14
5-7	WT	30.2 ± 2.5a	58.1 ± 5.3a	5.2a	16.2a	0.04 ± 0.01a	9
	TG	23.9 ± 2.1a	38.9 ± 2.2b	1.5a	17.7a	0.03 ± 0.01b	8
choice							
5-7	WT	15.7 ± 1.5a	41.6 ± 3.5a	NA	NA	NA	10
	TG	7.8 ± 1.0b	19.3 ± 2.0b	NA	NA	NA	10

[a] Feeding rate and weight values are means ± standard errors and are cumulative over time. Values in columns for like studies followed by different letters are statistically different at $P < 0.05$ by chi square (mortality) or ANOVA (feeding rates and survivor weights). Mortality values are based on found larvae. Due to cannibalism of dead larvae, day 2 mortality values may be lower than day 1 values. WT, wild type; TG, transgenic; NA, not applicable.

mortality rate of larvae fed transgenic R_3 plant leaf disks was significantly greater than that for larvae fed wild type leaf disks in some cases, and surviving larvae were significantly smaller in most cases (**Table 3**). On the basis of choice assays, the caterpillars did not appear to sense the presence of the RIP because relative feeding ratios were the same on RIP versus non-RIP R_3 leaf disks as for no-choice assays run concurrently from the same leaves. Feeding damage was significantly negatively correlated with RIP levels visually estimated by comparison with standards for both 5-1 ($R = -0.475$, $P = 0.0001$) and 5-7 ($R = -0.591$, $P = 0.0001$) transformants, including wild type plants used in corresponding assays at 0 leaf RIP values (run only for younger plants). In several cases RIP quantities in leaves approached 100 ppm (see above).

Feeding by *L. serricorne* larvae was more difficult to interpret because larvae also bored into and fed within leaf veins; estimated equivalent vein volume to leaf area values were used. Feeding on both the 5-1 and 5-7 series of transgenic R_3 leaf disks was significantly reduced compared to feeding on wild-type leaf disks in some sets of assays for each transformant line (**Table 4**). The one set of 5-7 leaf disks that showed no significant differences in feeding between wild-type leaf disks was the same set that also showed no significant difference in feeding for *H. zea*. Mortality rates were not statistically different for larvae fed the leaf disks from the two types of plants, but weights of surviving larvae fed leaves from R_3 transgenic plants were significantly lower than those of larvae fed wild type leaf disks in one case and higher for another (**Table 4**). Feeding damage by *L. serricorne* larvae was significantly inversely correlated with RIP levels visually estimated by comparison with standards for 5-1 series leaves from young plants ($P = 0.015$, $R = -0.32$) when control plants with 0 RIP values were included. Although feeding damage was not significantly ($P > 0.05$) correlated with RIP levels for 5-7 series leaves from young plants, the same inverse trend between RIP levels and feeding was noted ($R = -0.24$).

Feeding by *M. sexta* larvae on leaf disks from older plants of both transformant R_3 lines was also reduced in several cases compared to feeding on wild type plant leaf disks on day 1, but significant differences did not occur (**Table 5**). No significant differences in mortality occurred for larvae fed one type of disk versus another. In one case, *M. sexta* larvae that fed on 5-7 transformant leaf disks weighed significantly more than those

Table 4. Effect of Wild Type and Transgenic Maize RIP Young Tobacco R_3 Plants on *L. serricorne*[a]

plant series		feeding rate (mm²)	% mortality		survivor wt (mg)	N
		day 7	day 4	day 6		
5-1						
set 1	WT	76.3 ± 13.9a	18.8a	12.0a	0.31 ± 0.03a	7
	TG	42.9 ± 5.1b	28.1a	25.5a	0.37 ± 0.04a	12
set 2	WT	46.4 ± 3.4a	7.2a	10.9a	0.45 ± 0.04a	15
	TG	23.6 ± 2.6b	10.6a	10.8a	0.46 ± 0.04a	15
set 3	WT	54.8 ± 7.1a	4.2a	25.0a	0.31 ± 0.03a	6
	TG	48.6 ± 6.7a	6.7a	29.6a	0.21 ± 0.04b	7
5-7						
set 1	WT	77.6 ± 8.0a	0.0a	0.0b	0.37 ± 0.04a	6
	TG	90.0 ± 16.1a	8.0a	9.5a	0.31 ± 0.03a	7
set 2	WT	46.6 ± 5.2a	3.8a	25.8a	0.22 ± 0.02a	16
	TG	30.2 ± 3.5b	1.2a	28.4a	0.33 ± 0.05b	17

[a] Feeding rate and weight values are means ± standard errors and are cumulative over time. Values in columns for like studies followed by different letters are statistically different at $P < 0.05$ by chi square (mortality) or ANOVA (feeding rates and survivor weights). Mortality values are based on found larvae. Due to cannibalism of dead larvae, day 2 mortality values may be lower than day 1 values. WT, wild type; TG, transgenic.

Table 5. Effect of Wild Type and Transgenic Maize RIP Older Tobacco R_3 Plants on *M. sexta*[a]

series		feeding rate		% mortality		survivor wt (mg)	plant N
		day 1 (mm²)	day 2 (tenths of disk)	day 1	day 2		
5-1	WT	33.1 ± 2.0a	5.3 ± 0.3a	0.0a	0.0a	6.9 ± 0.3a	15
	TG	26.2 ± 1.2a	5.3 ± 0.3a	0.0a	0.0a	7.1 ± 0.2a	15
5-7	WT	37.6 ± 3.1a	4.5 ± 0.2a	0.0a	0.0a	7.8 ± 0.3a	13
	TG	32.1 ± 1.8a	5.1 ± 0.2a	3.6a	3.6a	8.7 ± 0.2b	14
choice							
5-7	WT	29.4 ± 8.2a	7.3 ± 0.7a	NA	NA	NA	10
	TG	22.8 ± 7.2a	7.7 ± 0.4a	NA	NA	NA	10

[a] Feeding rate and weight values are means ± standard errors and are cumulative over time. Values in columns for like studies followed by different letters are statistically different at $P < 0.05$ by chi square (mortality) or ANOVA (feeding rates and survivor weights). Mortality values are based on found larvae. Due to cannibalism of dead larvae, day 2 mortality values may be lower than day 1 values. WT, wild type; TG, transgenic; NA, not applicable.

that fed on wild type leaf disks. As for *H. zea*, the *M. sexta* larvae apparently could not sense the RIP, because feeding ratios for RIP versus non-RIP plants were the same as for no-choice assays set up from the same plant leaves on the same date.

DISCUSSION

Initial assays using purified active RIP in defined insect diets indicated significant activity against *H. zea* at 1000 ppm (*7*). In the present study, we saw significant activity against *H. zea* when transformed *N. tabacum* R_2 leaf disks had RIP levels in the 100 ppm range. The level of resistance seen for the transgenic plants expressing RIP is reasonable. Past reports have indicated the toxicity of a particular material is often less when insects are fed on defined diets versus actual plant tissue. Artificial diets are highly nutritious compared to natural leaf tissue, and the presence of high nutrient levels may obscure effects that would otherwise be seen under natural nutrient levels (*22*). In addition, artificial diets are less likely to contain natural defensive compounds, which may act additively or synergistically with a test compound. In tobacco, toxic defensive compounds can include protease inhibitors (*23*), products that are the result of peroxidase or polyphenol oxidase activity (*18*),

and secondary metabolites such as rutin or chlorogenic acid (*24*, *25*) as well as alkaloids (*26*). In natural combinations, plant defensive compounds can potentiate activity of bioassayed materials and/or cause increased stress on the insect (*27*). Another reason for concluding that maize RIP in the transformed plants is the likely cause of increased *H. zea* resistance is the significant negative correlations seen between feeding ratings and RIP levels for both transgenic lines. It is possible that additional factors induced due to damaging the plants by removing the leaf for initial bioassays would obscure some of the RIP effects on insects in assays with the older plants. However, assays with *H. zea* still indicated significantly less feeding on transgenic versus wild type leaf disks, although feeding on the disks from older plants was generally less for both plant types relative to the younger plants. Similar trends also showing reduced feeding by *H. zea* larvae on older plants occurred in studies with tobacco and tomato expressing high levels of tobacco anionic peroxidase (*18*, *20*).

On the basis of prior studies, we thought that the RIP-producing plants may have been more active against *M. sexta* than against *H. zea*. In our earlier studies, activated maize RIP was least effective against Indian meal moth larvae [*Plodia interpunctella* (Hübner)], which feed on mature kernels that have naturally high levels of RIP (*7*). The activated maize RIP was most active against cabbage looper larvae [*Trichoplusia ni* (Hübner)], which do not feed on maize at all (*7*). It is possible that the much larger size of the *M. sexta* versus *H. zea* larvae caused the relatively less sensitive response to the activated maize RIP. The larger larvae of *H. zea* are relatively unaffected by plants overexpressing tobacco anionic peroxidase, compared to newly hatched larvae (*18*, *20*, *28*). It is also possible that because the *M. sexta* studies were done with leaves from only the older, previously damaged R$_3$ plants, additional induced resistance factors may have proportionally been more important and equivalent in the transgenic and wild type plants compared to RIP. This would also explain why resistance to *M. sexta* was seen in some R$_2$ plants, but not R$_3$.

Our results indicate the maize RIP gene can be introduced into nonsource plants and enhance resistance of different insect species to various degrees. Higher levels of expression are probably necessary to obtain as complete resistance as occurs against European corn borers [*Ostrinia nubilalis* (Hübner)] in commercial hybrids of maize (e.g., refs *29* and *30*). Comparing the present plant-based study with our prior artificial diet-based study (*7*) and levels of RIP needed to attain similar activity, interactions with the natural plant environment may result in combinations that are more effective than predicted by assays on artificial diet (*22*). At present expression levels, the maize RIP may be useful as a component of a multigenic strategy for insect pest management, thereby reducing losses due to insect damage and their contribution to the indirect production of mold toxins in susceptible crops. Many insect species can contribute to the mycotoxin problem in maize, including *H. zea* and other caterpillar species, as well as different beetle species (*1*).

ABBREVIATIONS USED

RIP, ribosome-inactivating protein; CTAB, cetyltrimethyl-ammonium bromide; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; MSMO, Murashige and Skoog basal salts with minimal organics; PVDF, polyvinyldifluoride; TST, buffer containing 0.01 M Tris, 0.8% (w/v) NaCl, and 0.1% (v/v) Tween-20; HRP, horseradish peroxidase; DTT, dithiothreitol; PVP, polyvinylpyrrolidone; PAG, polyacrylamide gel; PAGE, polyacrylamide gel electrophoresis.

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