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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*

PATRICK F. DOWD,*,† ROBERT A. HOLMES,‡ T. SCOTT PINKERTON,† ERIC T. JOHNSON,† L. MARK LAGRIMINI,§ AND REBECCA S. BOSTON‡

Crop BioProtection Research Unit, National Center for Agricultural Utilization Research, Agricultural Research Service, U.S. Department of Agriculture, 1815 North University Street, Peoria, Illinois 61604, Department of Botany, North Carolina State University, Campus Box 7612, Raleigh, North Carolina, 27695-7612, and Department of Agronomy and Horticulture, University of Nebraska−Lincoln, Lincoln, Nebraska 68583

Tobacco (*Nicotiana tabacum*) plants grown from seed obtained by crossing a tobacco line that expressed an activated maize ribosome-inactivating protein (RIP) with a line that overexpressed tobacco anionic peroxidase were tested for their effects on corn earworm *Helicoverpa zea* and cigarette beetle *Lasioderma serricorne* larvae as compared to the wild-type plant cross. Significant feeding reductions were noted for transgenic plants expressing both resistance proteins as compared to wild-type plants for both *H. zea* and *L. serricorne*. Significant increases in mortality were also noted for those insects fed on the transgenic cross as compared to wild-type plants in some cases. Levels of both peroxidase and maize RIP were significantly higher in transgenic as compared to wild-type plants (which did not produce maize RIP). The degree of feeding was significantly negatively correlated with the level of RIP or peroxidase individually.

KEYWORDS: ***Helicoverpa*; *Lasioderma*; peroxidase; ribosome-inactivating protein; insect resistance; transgenic**

INTRODUCTION

Insects cause billions of dollars of damage to crop plants and also promote the colonization of fungi that produce toxins and carcinogens (*1*, *2*). Enhanced insect resistance through genetic engineering has the potential to introduce novel resistance mechanisms that are not possible through conventional breeding. Many putative insect resistance genes have been tested for efficacy in transgenic plants. Of these, genetically modified varieties expressing different forms of the *Bacillus thuringiensis* (Bt) crystal protein are the most widely planted. The Bt lines have activity against different caterpillars and beetles. However, because of similarity of structure and mode of action of the Bt proteins, there is concern that insects will develop broad spectrum resistance to them (e.g., *3*, *4*). Laboratory selection studies have confirmed these concerns. Both altered target site and enzymatic degradation have been noted as resistance mechanisms that are effective across different classes of Bt proteins (*6*).

Combinations of plant resistance genes are thought to delay the development of resistance, a concept that is based on many years of studies with insecticides (e.g., *3*). This approach has been tested by combining the gene encoding the Bt crystal protein with other genes. In at least one such laboratory study, a delay in the development of resistance has been reported (*6*).

It should also be possible to combine plant-derived genes to generate more effective and stable resistance to insects. Multiple resistance genes occur naturally in individual species of plants (*7*) and fungi (*8*). However, it may be necessary to determine compatibility of different resistance mechanisms. For example, proteases involved in insect resistance may degrade other resistance proteins. Protease inhibitors may inhibit not only proteases but also other hydrolytic enzymes with functionally similar active sites involved in resistance, such as chitinases.

We have examined two plant-derived insect resistance proteins with greatly different modes of action, both of which appear to be active against different groups of insects (i.e., caterpillars and beetles). Tobacco anionic peroxidase (POX) appears to have multiple modes of action, which are dependent on the plant species and tissue in which it is expressed (*9*).

* To whom correspondence should be addressed. Tel: 309-681-6242. Fax: 309-681-6686. E-mail: dowdpf@ncaur.usda.gov.
† U.S. Department of Agriculture.
‡ North Carolina State University.
§ University of Nebraska−Lincoln.

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Transgenic plants expressing high levels of tobacco anionic POX have resulted in reduced numbers and feeding by insects in five different groups as compared to wild-type plants: caterpillars, beetles, whiteflies, aphids, and grasshoppers (*10*−*14*). Maize ribosome-inactivating protein (RIP), an RNA N-glycolyase, specifically depurinates an adenine base in large ribosomal RNA, which results in reduced protein synthesis (*15*−*17*). Activated maize RIP has activity against several caterpillar species that are pests of corn (*18*) as well as beetles (*19*). Because of their very different modes of action, producing these two proteins at high levels in the same plant would be of potential interest for dual targeting and as a means of reducing the rate at which insects may develop resistance to them. However, POX activity can result in multiple functionalities, including protein binding or inhibition of hydrolytic enzymes (*12*, *13*, *20*), which could lead to incompatibility with other resistance proteins such as maize RIP. The present study was initiated to evaluate plants expressing both of these proteins, since when expressed individually each has led to increased insect resistance (*12*, *19*, *21*). Homozygous plants that either overexpressed tobacco anionic POX or expressed maize RIP were crossed to produce hybrid plants with both transgenes. The resulting hybrids were tested in bioassays for resistance to feeding by target insects as compared to wild-type crosses.

MATERIALS AND METHODS

Insects. The *Helicoverpa zea* were reared at 27 ± 1° C, 40 ± 10% relative humidity, and a 14:10 light:dark photoperiod on a pinto bean-based diet as described previously (*12*, *19*, *22*). The *Lasioderma serricorne* were reared under the same temperature, humidity, and light conditions on a flour/corn meal-based diet (*19*). Newly hatched first instar larvae of *H. zea* and second instar larvae of *L. serricorne* were used for bioassays.

Plants. The wild-type variety for the tobacco anionic POX transgenic plants was Coker 176, and the wild-type variety for the maize RIP tobacco plants was Kentucky 14. The transgenic tobacco line that overexpressed the tobacco anionic POX was 507C, which contains the gene in a homozygous state and has stably expressed 10× or higher levels of POX as compared to wild-type plants through successive generations (*12*, *21*, *23*). The construct consisted of a cauliflower mozaic virus (CaMV) 35S promoter, the plml507 tobacco anionic POX clone, and a NOS terminator (*24*, *25*). The transgenic line of tobacco that expressed the maize RIP gene was derived from lines 5−7 (*19*) and expressed up to 100 ppm of the protein on a wet weight basis (*19*). The construct consisted of the active RIP coding sequence excised from MOD1 and inserted between a CaMV 35S promoter and NOS terminator in a modified pBIN19 vector (*17*). Selection studies on MS media containing 300 ppm of kanamycin were used to determine that the maize RIP gene occurred in the homozygous state in the 5-7-7-8 plant, as greater than 90% of selfed seed that germinated survived at this concentration, while 50% or less survival was noted on most of the other selfed seed run concurrently on the same media. The maize RIP 5-7-7-8 line served as the female parent, and a 507C POX plant was used as the pollen source to obtain crosses. Wild-type crosses between Coker 176 and KY14 were made in the same manner. Plants were crossed in a plant growth facility held at 50 ± 10% relative humidity, 14:10 light:dark cycle, with a day temperature of 24 ± 1° C and a night temperature of 18 ± 1° C.

Seed from crossed plants, as well as individual wild-type plants, was germinated at 27 ± 1° C, 40 ± 10% relative humidity, and a 14:10 light:dark photoperiod in RediEarth (Scotts Sierra Horticultural Products, Marysville, OH) as described previously (*12*, *19*). Plants were transplanted to individual 10 cm square pots when they reached a four leaf stage, and a spike of 6-12-6 nitrogen−phosphorus−potassium (NPK) time release fertilizer (Miracle-Gro Indoor Plant Food Spike, Scotts Miracle Gro Products Inc., Milpitas, CA) was added to each pot.

Bioassays. Leaves were removed for bioassays when the plants were approximately 2 weeks old, with the largest leaf about 4 cm in diameter. The largest fresh leaf (typically the second leaf from the terminal one) was removed from each plant. Two 0.8 cm diameter disks were removed from either side of the vein at the tip of the leaf for subsequent protein determinations, and a single 2 cm diameter leaf disk was removed immediately below this location for use in bioassays. Approximately 1 week later, a second series of bioassays was set up with larger leaves (ca. 8−10 cm in diameter) from the same relative position on the same plants as described for the first series. The two smaller leaf disks were removed for future protein assays as just described, and a 2 cm disk was removed on either side of the midvein for use in bioassays. Individual leaves from at least 12 plants of each type were used in each series of assays.

Bioassays were run in 5 cm diameter Petri dishes with tight-fitting lids (Falcon 1006, Becton Dickinson, Lincoln Park, NJ) as described previously (*12*, *19*). Ten first instar *H. zea* or five second instar *L. serricorne* were added to each leaf disk. Feeding damage was determined visually by estimating the number or equivalent area of 0.25 mm^2 holes (approximately head capsule size) and 1 mm^2 holes chewed in the disk by the *H. zea* on the first and second days of study, respectively (*10*, *12*, *19*). Feeding damage by the *L. serricorne* larvae was determined similarly using the 1 mm^2 hole criteria on the second and fourth days of the study, as described previously (*19*). Mortality was also determined each day that leaf feeding was checked. After leaf rating was done on the final day, the insects were frozen and then weighed on an analytical balance (Mettler AE 163, Mettler Instrument Corp., Highstown, NJ) with a precision of 0.01 mg.

Protein and Nucleic Acid Determinations. The POX activity was determined spectrophotometrically using pyrogallol as a substrate as described previously (*12*, *21*, *26*). The leaf disk was homogenized in 0.5 mL of ice cold, pH 6.0, 0.1 M phosphate buffer using a ground glass homogenizer. The homogenate was centrifuged at 10000*g* for 10 min, and the supernatant was used as the enzyme source. The concentration of the enzyme was adjusted to give a linear response curve over the monitoring period (2−10 min) at 420 nm using a Spectramax 250 spectrophotometer (Molecular Devices Corp., Sunnyvale, CA), with 0.28% pyrogallol (Sigma-Aldrich Corp., St. Louis, MO) and 0.3% hydrogen peroxide at pH 6.0. Homogenates were also examined for production of the appropriate POX isozyme by isoelectric focusing as described previously (*26*). Maize RIP was detected on sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) Western blots probed with antibody raised against purified proRIP as described previously (*19*), with a dithiothreitol concentration of 1 mM. Different quantities of purified recombinant MOD1 expressed in *Escherichia coli* were used as standards (*19*). The MOD1 is 4.2 kDa larger than the RIP produced in the transgenic plants. Quantities of RIP present were determined from standardized MOD1 bands using ImageMaster 1D software (Amersham Pharmacia Biotech, Piscataway, NJ).

The presence of the transgenes was confirmed by polymerase chain reaction (PCR) analysis with at least 200 ng of genomic DNA, using procedures described previously (*19*, *27*). The same forward primers, which occurred in the CaMV35S promoter, were used to detect both the RIP and the POX constructs: 5′-atgacgcacaatcccacta-3′. The reverse complement primer used to detect the maize RIP gene (internal to the gene) was 5′-gcagcagatcatgatgtgtc-3′. The reverse complement primer used to detect the tobacco anionic POX gene (internal to the gene) was 5′-tgccacgacggacctttagc-3′. The PCR program specified an initial denaturation step at 94 °C for 3 min followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min and then a final extension for 10 min at 72 °C, using model PTC-150 minicycler (MJ Research, Waltham, MA). PCR products were detected as described previously (*27*) except that 1% agarose gels were used. At least four representative plants of wild-type and transgenic crosses from each set were used in the analyses. Representative PCR products from two different transgenic plants were isolated using the Geneclean kit (Qbiogene, Irvine, CA) and then cloned into the TOPO TA Cloning vector (Invitrogen, Carlsbad, CA). Plasmid DNA isolated from the transformants was sequenced using the BigDye Terminator 3.1 Cycle

Table 1. Effect of Combined Transgenic Expression of Tobacco Anionic POX and Maize RIP in Tobacco on *H. zea*[a]

plant type	day 1 % mortality	day 1 feeding (0.25 mm^2)	day 2 % mortality	day 2 feeding (1.0 mm^2)	weight of surviving larvae (mg)
small leaf set 1					
KY14	8.7 a	31.8 ± 2.4 a	5.6 a	31.0 ± 2.6 a	ND
Coker 176	10.9 a	32.4 ± 3.0 a	4.1 a	30.0 ± 2.3 a	ND
KY × Coker	7.2 a	31.9 ± 2.0 a	2.7 a	35.9 ± 3.1 a	0.23 ± 0.01 a
RIP × POX	12.0 a	21.2 ± 2.1 b	6.0 a	24.8 ± 1.9 b	0.26 ± 0.01 a
small leaf set 2					
KY14	20.7 a	19.8 ± 1.6 a	9.3 a	26.0 ± 1.9 a	ND
Coker 176	18.0 a	23.8 ± 1.4 a	8.1 a	27.1 ± 1.4 a	ND
KY × Coker	13.5 a	24.1 ± 1.8 a	8.2 a	30.1 ± 2.5 a	0.21 ± 0.01 a
RIP × POX	24.8 b	14.6 ± 1.6 b	13.2 a	23.2 ± 1.8 b	0.18 ± 0.01 a
large leaf set 1					
KY × Coker	57.0 a	10.2 ± 0.9 a	57.5 a	10.0 ± 1.0 a	0.21 ± 0.02 a
RIP × POX	67.0 a	5.7 ± 0.5 b	56.0 a	5.8 ± 0.7 b	0.24 ± 0.02 a
large leaf set 2					
KY × Coker	22.0 a	14.9 ± 1.5 a	12.0 a	9.1 ± 0.9 a	0.09 ± 0.01 a
RIP × POX	38.9 b	5.1 ± 0.6 b	42.4 b	4.1 ± 0.7 b	0.09 ± 0.01 a

[a] Feeding rate and surviving larvae weight values are means ± standard errors for assays with at least 12 leaf disks. Values of like studies in columns followed by different letters are statistically different as compared to the wild-type cross by ANOVA (feeding and weight values) or χ^2 (mortality values) at $P < 0.05$; ND, not determined.

Sequencing Kit (Applied Biosystems, Foster City, CA) and an ABI 3100 sequencer (Applied Biosystems).

Statistics. Significant differences in mortality or other percentage data were determined by χ^2 analysis using SAS program PROC FREQ (*28*). Significant differences in weights or feeding rates were determined by analysis of variance (ANOVA) using SAS program PROC ANOVA or PROC GLM (*28*). Significant correlations between feeding or weight responses and POX or RIP concentrations, or a combination thereof, were determined using SAS program PROC REG (*28*). MAXR iterative variable substitution was used when both POX and RIP were used as variables at the same time (*28*).

RESULTS

On both day one and day two, damage of the RIP × POX leaf disks from smaller leaves by *H. zea* was significantly less as compared to the KY14, Coker 176, or hybrid wild-type leaf disks for sets one and two (**Table 1**). Feeding on leaf disks from individual wild-type lines was not significantly different from wild-type crosses, but in several cases, the trend was for higher rates of feeding on the wild-type cross. On day one, the mortality of larvae in the second set fed RIP × POX disks was significantly higher ($P = 0.022$, $\chi^2 = 5.207$) than for larvae fed the wild-type cross KY14 × Coker 176 disks (**Table 1**). There were no additional significant differences in mortality between larvae fed on any of the leaf disk types in either set for either day of the study, although the general trend was for higher mortality for larvae fed the RIP × POX leaf disk fed larvae as compared to that for larvae fed wild-type KY14 × Coker 176 leaf disks. Weights of surviving larvae fed the RIP × POX disks were not significantly different from those of larvae fed other leaf disks at $P < 0.05$.

On days one and two, damage of the RIP × POX disks from larger leaves by *H. zea* was significantly less than that of the hybrid wild-type leaf disks for sets one and two (**Table 1**). Mortality of larvae fed the RIP × POX leaf disks was significantly higher than that for the wild-type cross in set two of the assays both on day 1 ($P = 0.011$, $\chi^2 = 6.434$) and day 2 ($P < 0.0001$, $\chi^2 = 16.088$). Although there were no significant differences in mortality of larvae fed RIP × POX leaf disks vs wild-type KY14 x Coker 176 disks for the first set of assays with larger leaves, the same trend for higher mortality was noted for those larvae fed the RIP × POX disks vs wild-type cross disks on day 1. Because of cannibalism of dead larvae, in some cases, mortality is lower for day 2 than day 1. Weights of surviving larvae fed the RIP × POX disks were not significantly different as compared to those fed the wild-type leaf disks at $P < 0.05$.

Table 2. Effect of Combined Transgenic Expression of Tobacco Anionic POX and Maize RIP in Tobacco on *L. serricorne*[a]

plant type	day 2 % mortality	day 2 feeding (1.0 mm^2)	day 4 % mortality	day 4 feeding (1.0 mm^2)	weight of surviving larvae (mg)
large leaf set 1					
KY × Coker	2.7 a	10.5 ± 1.1 a	1.4 a	10.3 ± 1.0 a	1.1 a
RIP × POX	1.4 a	6.3 ± 0.7 b	1.4 a	6.2 ± 0.8 b	1.2 a
large leaf set 2					
KY × Coker	1.5 a	5.6 ± 1.0 a	6.5 a	7.4 ± 1.3 a	0.5 a
RIP × POX	8.3 a	3.0 ± 0.5 b	18.6 b	3.8 ± 0.7 b	0.5 a

[a] Feeding rate and surviving larvae weight values are means ± standard errors for assays with at least 12 leaf disks. Values of like studies in columns followed by different letters are statistically different by ANOVA (feeding and weight values) or χ^2 (mortality values) at $P < 0.05$.

On days two and four, damage of the RIP × POX leaf disks from larger leaves by *L. serricorne* was significantly less as compared to the hybrid wild-type plants for sets one and two (**Table 2**). The *L. serricorne* larvae of set 2 fed RIP × POX leaves had significantly higher mortality on day 4 ($P = 0.028$, $\chi^2 = 4.805$) as compared to larvae fed the wild-type KY14 × Coker 176 leaf disks. There were no significant differences in mortality between larvae fed on any of the other leaf disk types in either set, but the same trend for higher mortality of larvae fed leaf disks from the RIP × POX plants vs wild-type plants was noted for larvae in the second set of assays on day 4 (which were also smaller sized overall than the first set of larvae). There were no significant differences at $P < 0.05$ in weights of surviving larvae fed the RIP × POX disks vs wild-type KY14 × Coker 176 leaf disks in either set of assays.

Quantitation of RIP indicated that all plants expected to produce RIP did produce RIP, in the range of approximately 5−35 ng/mg of leaf tissue for both the smaller and the larger leaves (**Table 3** and **Figure 1**). There was typically a significant inverse correlation between RIP content of the leaf and feeding rate of *H. zea* in assays with the smaller and larger leaves in both sets (**Table 4**). Likewise, there was typically a significant inverse correlation between RIP content of the leaf and feeding rate of *L. serricorne* in assays with the larger leaves in both sets (**Table 4**).

Quantitation of POX activity indicated that all plants expected to produce higher POX than wild-type plants did produce higher levels of POX, in the range of 50−100× for the smaller leaves and 15−20× for larger leaves, with the tobacco anionic POX being the major POX produced (**Table 3** and **Figure 2**) in both sets. There was typically a significant inverse correlation between POX activity of the leaf tissue and feeding rate of *H. zea* in assays with the smaller and larger leaves in both sets, with some tendency for the correlation to be lower in day 2 vs day 1 of the assays (**Table 4**). There was typically a significant inverse correlation between POX activity of the leaf and feeding rate of *L. serricorne* in assays with the larger leaves in both sets (**Table 4**). When both RIP and POX were considered together in correlation analyses, there was no obvious trend for one to be more strongly correlated with the feeding rating than

Table 3. POX and Maize RIP Expression in Transgenic Tobacco Plants[a]

variety	maize RIP (ng/mg leaf tissue)	POX (Δ absorbance units/10 min/leaf disk)
	small leaf set 1	
KY14	0[b]	0.02 ± 0.01[b]
Coker 176	0[b]	0.10 ± 0.02[b]
KY × Coker	0[b]	0.04 ± 0.02
RIP × POX	15.1 ± 0.7	4.80 ± 0.50
	small leaf set 2	
KY14	0[b]	0.47 ± 0.21[b]
Coker 176	0[b]	0.50 ± 0.09[b]
KY × Coker	0[b]	0.10 ± 0.03
RIP × POX	18.1 ± 1.0	5.20 ± 0.30
	large leaf set 1	
KY × Coker	0[b]	0.50 ± 0.20
RIP × POX	12.0 ± 0.4	7.90 ± 0.60
	large leaf set 2	
KY × Coker	0[b]	0.50 ± 0.20
RIP × POX	13.5 ± 0.9	9.10 ± 0.30

[a] Values are means ± standard errors. Means are derived from at least 12 values for each of the crosses and three values for each of the individual varieties. Mean weights of leaf disks were approximately 10 mg for the small leaf set and 13 mg for the large leaf set. [b] Representative samples.

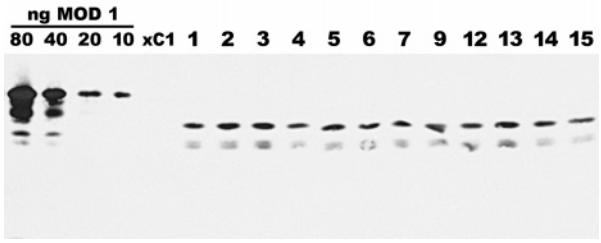

Figure 1. Detection of maize RIP in transgenic plant crosses by SDS–PAGE Western blot antibody analysis. MOD1 is the RIP standard, and XC1 is the wild-type cross plant. Other numbered lanes are different transgenic plants. Slightly smaller bands in respective lanes are what would be expected if the carboxy terminal portion had been removed (*19*).

Table 4. Correlation Analysis between Transgene Protein Contents and Biological Activity (Feeding Rate) of Tobacco Hybrids[a]

	day 1		day 2	
insect assay	CC	*P* value	CC	*P* value
	POX			
HZ small leaf set 1	–0.46	0.011	–0.43	0.016
HZ small leaf set 2	–0.58	0.001	–0.32	0.033
HZ large leaf set 1	–0.60	0.004	–0.47	0.0082
HZ large leaf set 2	–0.78	<0.0001	–0.66	0.0001
LS large leaf set 1	–0.43	0.017	–0.46	0.010
LS large leaf set 2	–0.39	0.042	–0.42	0.027
	RIP			
HZ small leaf set 1	–0.41	0.016	–0.36	0.0684
HZ small leaf set 2	–0.58	0.0013	–0.34	0.0738
HZ large leaf set 1	–0.67	<0.0001	–0.58	0.0012
HZ large leaf set 2	–0.74	<0.0001	–0.68	0.0002
LS large leaf set 1	–0.50	0.0071	–0.55	0.0025
LS large leaf set 2	–0.37	0.059	–0.39	0.049

[a] HZ, *H. zea*; LS, *L. sericorne*; and CC, correlation coefficient.

the other, and in most cases, only a slight improvement in the correlation was noted when both variables were used in the regression analysis (data not shown).

Both maize RIP (**Figure 3a**) and tobacco anionic POX (**Figure 3b**) transgene constructs were detected by PCR analysis

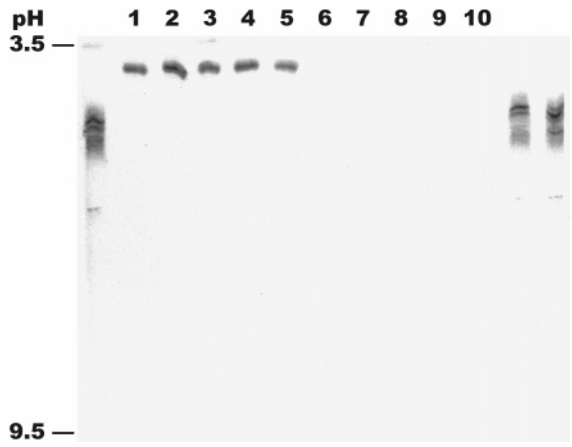

Figure 2. Detection of tobacco anionic POX in transgenic plant crosses by isoelectric focusing and direct staining. Lanes 1–5 are transgenic plants 1–5, and lanes 6–10 are wild-type plants 1–5. Unnumbered lanes are the marker dye Evan's blue.

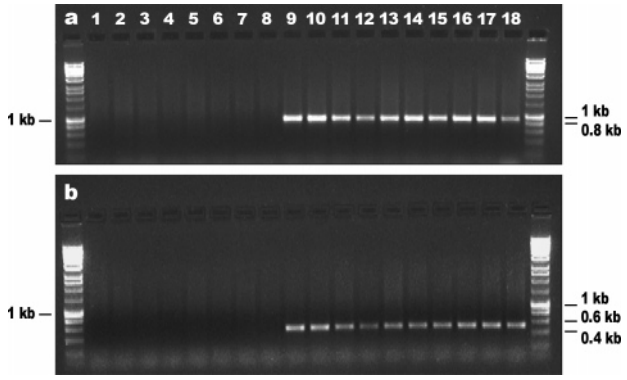

Figure 3. PCR detection of maize RIP gene construct (**a**) and tobacco anionic POX gene construct (**b**) in transgenic plant crosses. Lanes 1–8, wild-type cross samples from plants 1–3, 6, 17, 22, and 23. Lanes 9–18, transgenic cross samples from plants 1–5 and 16–20. The RIP product is ca. 900 bp, and the POX product is ca. 500 bp. Unnumbered lanes are molecular weight markers.

in the representative plant samples that were examined. The sequences of the PCR products were identical to the corresponding sequences of the inserted transgenes (data not shown).

DISCUSSION

Prior studies have indicated an association between elevated POX or different types of RIP levels and activity against insects (*11*, *12*, *29*, *30*). Our past studies with transgenic plants have indicated significant increases in mortality and reduced weights of survivors in some cases and significant reductions in feeding in most cases, when insects were fed on plant tissues expressing higher levels of tobacco anionic POX or maize RIP. The range of feeding reduction and weight reduction for *H. zea* fed Coker 176 tobacco plants with higher levels of POX was generally 1.5−2.0-fold, with significant differences in mortality in some cases (*12*, *21*). The range of feeding reduction and weight reduction for *L. serricorne* fed Coker 176 tobacco plants with higher levels of POX was generally 1.5−2.0-fold (*12*, *21*). The range of feeding reduction and weight reduction for *H. zea* and *L. serricorne* fed KY14 tobacco plants expressing maize RIP was generally 1.5−2.0-fold, with some significant differences in mortality for *H. zea* but not *L. serricorne* (*19*). In these studies, the dose of the tobacco anionic POX gene would have been 2× (homozygous condition) but the dose of the maize RIP

gene would have primarily been 1× (heterozygous condition) as subsequently only a few of the 5-1 series and 5-7 series RIP-expressing tobacco plants were found to be homozygous (Dowd et al. Unpublished data). In both studies, the activity of the POX (*21*) and maize RIP (*19*) was significantly inversely correlated with the expression levels of the respective defensive protein, as was generally noted in the present study (**Table 4**). Thus, the activity in the present study, where both genes would have been in 1× state, appeared to correspond to what would have been expected for *H. zea* and *L. serricorne* on an additive basis. On the basis of comparisons of individual wild-type lines and the wild-type crosses, there appeared to be no significant hybridization effect. More definitive studies would be required to determine if more subtle antagonism or synergism occurred.

Further improvement in insect control may be related to a need to overcome some other hurdle for efficacy. For example, the activity of the proteins or products produced in the present study may be limited by the rate of penetration to the target site by the peritrophic membrane lining the insect gut. Removal of this or other barriers may result in synergistic activity. For example, coadministration of chitinase with Bt crystal protein has resulted in synergized toxicity to some species of caterpillars (*31*, review). Apparently, the chitinase produced holes in the peritrophic membrane lining the insect gut, resulting in more ready passage of the Bt crystal protein to the target gut epithelial tissue (*31*, review).

In the present study, activity of transgenic vs wild-type crosses against insects appeared to be greater for the larger leaves as compared to the smaller leaves. This activity trend has also been reported in previous studies for high POX plants (*21*). Insect damage can induce different defensive proteins or enzymes involved in the biosynthesis of defensive secondary metabolites (*32*, *33*). It is possible that these induced compounds act additively or synergistically with the constitutively produced transgenic proteins. However, the defensive role of secondary metabolites induced in tobacco based on earlier in vitro studies appears less important in studies where similar levels of metabolites were expressed constitutively in transgenic plants (*34*).

The second series of studies reported here showed greater activity against *L. serricorne* than the first set of studies. On the basis of final weights, it appears larvae that were used in the second set of assays were smaller than those used in first set. These data are consistent with our previous observations that larger insect instars are less susceptible to the effects of the POX expressed in transgenic plants as compared to smaller instars (*9*−*12*). Past studies (*10*−*12*, *19*) have sometimes indicated significantly smaller weights of surviving larvae fed high POX or maize RIP, in contrast to the present study, where no significant differences in weights of survivors were noted. It is possible that in some cases, insects that died in the present study would be those that otherwise would have survived and thus would contribute to lower mean weights had they survived and been included in the insects that were weighed. It is also possible that some interference between the two resistance mechanisms occurred, especially for *H. zea*.

Interference between different resistance mechanisms has been reported previously. For example, condensed tannins, secondary metabolites that can bind proteins, interfere with the activity of the Bt crystal protein CryIA(c) toward *Heliothis virescens* (*35*). It is uncertain what type of interference might have occurred in the present study. As mentioned in the Introduction, POX activity can result in the formation of protein complexes, depending on the cofactors that are present (*13*, *20*). However, in the present study, there appeared to be no obvious antagonism or synergism as compared to results of prior studies where transgenic plants contained only one introduced gene, as previously discussed.

Thus, our results indicate that tobacco plants expressing both maize RIP and elevated levels of tobacco anionic POX together were damaged significantly less by both a caterpillar (*H. zea*) and a beetle (*L. serricorne*). There was no apparent antagonism of the two introduced resistance mechanisms as compared to prior studies where the genes were examined individually. These genes may be useful components of a multigenic insect control strategy, which can potentially reduce insect damage and associated mycotoxins in susceptible crops.

ABBREVIATIONS USED

Bt, *Bacillus thuringiensis*; CaMV, cauliflower mozaic virus; NPK, nitrogen−phosphorus−potassium; PCR, polymerase chain reaction; POX, peroxidase; RIP, ribosome-inactivating protein; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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