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Modification of the coding sequence enhances plant expression of insect control protein genes

(*Bacillus thuringiensis*/insect-resistant plants/synthetic genes)

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ABSTRACT **Increased expression of the insect control protein genes of *Bacillus thuringiensis* in plants has been critical to the development of genetically improved plants with agronomically acceptable levels of insect resistance. The expression of the *cryIA(b)* gene was compared to partially modified (3% nucleotide difference) and to fully modified (21% nucleotide difference) *cryIA(b)* and *cryIA(c)* genes in tobacco and tomato. The modified genes increased the frequency of plants that produced the proteins at quantities sufficient to control insects and dramatically increased the levels of these proteins. Among the most highly expressing transformed plants for each gene, the plants with the partially modified *cryIA(b)* gene had a 10-fold higher level of insect control protein and plants with the fully modified *cryIA(b)* had a 100-fold higher level of CryIA(b) protein compared with the wild-type gene. Similar results were obtained with the fully modified *cryIA(c)* gene in plants. Specific sequences of the partially modified *cryIA(b)* gene were analyzed for their ability to affect *cryIA(b)* gene expression in tobacco. The DNA sequence of a single region was identified as important to the improvement of plant expression of the *cryIA(b)* gene. The increased levels of *cryIA(b)* mRNA were not directly proportional to the increased levels of CryIA(b) protein in plants transformed with the modified *cryIA(b)* genes, indicating that the nucleotide sequence of these genes had an effect in improving their translational efficiency in plants.**

Insect control proteins from a prokaryotic source, *Bacillus thuringiensis* var. *kurstaki* (*B.t.k.*; ref. 1) are specific for lepidopteran insects and exhibit no activity against humans, other vertebrates, and beneficial insects (2). These properties have made the genes of these insect-specific proteins attractive candidates for genetic modification of crops for protection against lepidopteran pests. Genes encoding lepidopteran-specific insect control proteins have been cloned and sequenced. Truncated genes, which produce insecticidally active protein, have been expressed in tomato (3), tobacco (4), and cotton (5). Field tests of these plants revealed that higher levels of insect control protein in the plant tissue would be required to obtain commercially useful plants (6).

The insect control proteins are highly expressed in their natural host, *B. thuringiensis*. Up to 50% of the total protein in sporulated cultures of *B.t.k.* are the insect control proteins deposited as crystals within the cell. Insect control protein genes are expressed well in *Escherichia coli* (7) or *Pseudomonas* (8). Poor expression in plants is a well-reported characteristic of the *B.t.k.* insect control proteins. Truncating the gene, keeping essentially the N-terminal half of the protein intact, results in improved expression of the gene in plants to barely detectable levels (3, 4). The use of different promoters, fusion proteins, and leader sequences has not significantly increased insect control protein gene expression (4, 9).

We hypothesized that a gene with a sequence adapted for a Gram-positive prokaryote may not be the appropriate coding sequence for efficient plant expression. Examination of the insect control protein gene coding sequence indicated that it differs significantly from plant genes in G+C content. Multiple sequences motifs that are not common in the coding region of plant genes were found to be common in the wild-type (WT) *cryIA(b)* sequence. These included localized regions of A+T richness resembling plant introns (10), potential plant polyadenylylation signal sequences (11), ATTTA sequences, which have been shown to destabilize mRNA in other systems (12), and codons rarely used in plants (13).‡

Two approaches were initiated to increase the level of CryIA(b) and CryIA(c) insect control proteins in genetically modified plants. First, DNA sequences predicted to inhibit efficient plant gene expression at both the translational and mRNA level were selectively removed throughout the coding sequence by site-directed mutagenesis to partially modify the gene (PM gene) without changing the amino acid sequence. Wholesale changes in the DNA characterized the second approach, which required the use of a fully modified (FM) synthetic gene. The FM genes encode proteins nearly identical in amino acid sequence to the WT gene. These constructs were a more rigorous application of the principles used to generate the PM gene, taking into account additional factors such as codon usage in plants, potential secondary structure of mRNA, and potential regulatory sequences. Insect control protein levels in plants increased as a consequence of modification of the DNA sequence, up to 100-fold over levels seen with the wild-type gene in plants. The increased gene expression appears to be the result of increased translational efficiency. The higher level of insect control protein in these plants was directly correlated to increased insecticidal activity. The demonstration of the utility of these genes to provide protection from insects has far-reaching implications for the future of insect-resistant plants and for the application of these gene modification principles to the design of heterologous genes for high-level expression in plants.

MATERIALS AND METHODS

Modifications of the Coding Sequence of Insect Control Genes. The coding sequence of the WT gene in pMON9921

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Abbreviations: WT, wild type; PM, partially modified; FM, fully modified; THW, tobacco hornworm; *B.t.k.*, *Bacillus thuringiensis* var. *kurstaki*.

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‡The sequence reported in this paper has been deposited in the GenBank data base (accession no. M60856).

was identical in DNA sequence and similar to the truncated version of *cryIA(b)* (3). Modifications in the DNA did not alter the amino acid sequence of the CryIA(b) protein. Partial modification of the WT sequence was by site-directed mutagenesis (14) with 9 oligonucleotides, labeled A–I as they appear in the sequence. The nucleotides that differ from the WT sequence are shown in Fig. 1. Regions of the coding sequence that were targeted for modification were identified by scanning the DNA sequence for 4 or more consecutive adenine (A) or thymine (T) nucleotides. These regions were analyzed for the presence of potential polyadenylylation signal sequences (11). If there was more than 1 signal sequence within 10 nucleotides, the sequence was altered. If any region of 15–30 nucleotides was >80% A+T, it was altered if there were 2 or more ATTTA sequences or if there was a major potential plant polyadenylylation signal sequence (AATAAA or AATAAT). Nucleotides used to modify the DNA sequence increased the overall G+C content, increased plant preferred codons (13) without changing the amino acid sequence, and did not generate consecutive A+T or G+C strings (>6).

The changes of the FM gene [FM *cryIA(b)*] required complete synthesis (British Biotechnology, Oxford, U.K.). The DNA sequence was designed to maintain the amino acid sequence and avoid ATTTA sequences and potential polyadenylylation signal sequences. Plant preferred codons were used. Consecutive strings of A+T or G+C nucleotides of 5 or more were avoided. The FM *cryIA(b)* gene is compared to the WT in Fig. 1. The FM *cryIA(c)* gene (5) is a synthetic truncated version of the *cryIA(c)* gene and encodes amino acids 1–615. A second FM *cryIA(c)* gene, described in Table 2, encodes amino acids 29–615.

Plant Expression Vectors. The genes for insect control were delivered as cointegrates of a disarmed nopaline plasmid (15). These intermediate vectors all contain the insect control protein genes [WT, PM, FM, or FM *cryIA(c)*] driven by the cauliflower mosaic virus 35S promoter with a duplicated enhancer region (16).

Plant Transformation and Regeneration. *Agrobacterium tumefaciens* was used to obtain transgenic tobacco (17) and tomato (18) plants.

Immunological Analysis. Western blot analysis was done as described (5). Antibodies prepared in rabbits to truncated, denatured insect control protein [trypsin-treated CryIA(c) protein] isolated from a SDS/polyacrylamide gel were used. The blots were sensitive to 1–2 ng per 50 μg of total plant protein. ELISA analysis of transgenic plant material for CryIA(b) protein utilized a direct double antibody sandwich with rabbit polyclonal antibody raised to the *cryIA(b)* gene product as expressed in *E. coli*, purified as described (19). The ELISA was sensitive to 0.5 ng per 50 μg of total protein.

mRNA Analysis. S1 nuclease protection assays (20) were used to quantitate the levels of *cryIA(b)*-specific mRNA. A ^{32}P kinase-labeled 45-mer synthetic oligonucleotide with 30 nucleotides of homology to the 5′ untranslated sequence common to all of the insect control protein gene plant mRNAs was hybridized with 40 μg of total RNA. An internal control of a labeled 55-mer with 40 nucleotides of homology to the coding sequence of the endogenous tomato 3-*enol*-pyruvoylshikimate-5-phosphate synthase (EPSPS) mRNA (21) was included in each reaction mixture. Each lane was scanned with a laser densitometer for quantitation and was normalized with respect to the levels of endogenous EPSPS mRNA. Several exposures were scanned to account for film saturation.

Insect Bioassay. Five neonate larvae of tobacco hornworm (THW) were placed on a leaf of a plant that had been selected in tissue culture for kanamycin resistance. Insect control was defined as plant tissue that exhibited 100% mortality to THW and sustained no visible damage after 3 days.

RESULTS

Partial modification of the WT coding sequence focused on potential plant polyadenylylation signal sequences and ATTTA sequences in regions of high local A+T content. The resulting gene, referred to as the PM gene, contained a subset of the changes made in the FM gene (Table 1 and Fig. 1). The design of the FM gene eliminated ATTTA sequences, almost all potential plant polyadenylylation sequences, greatly increased the G+C nucleotide content of the gene, and minimized the use of rare plant codons. The PM gene encodes amino acids 29–607 of the CryIA(b) protein with an additional two amino acids, methionine and alanine, at the N terminus. The FM gene encodes amino acids 1–615. Both genes have been expressed in *E. coli* and the proteins show equivalent activity against lepidopteran insects (data not shown).

Insect Control Protein Levels in Genetically Modified Plants. The plants expressing the WT, PM, or FM genes were identified from the transformed kanamycin-selected plants by a primary screen bioassay against THW (Table 2). No observable damage to the leaf by THW indicated that ingestion of small amounts of leaf tissue by THW was sufficient to kill the insects. The frequency of transgenic plants that exhibited insect control was higher in the PM and FM transformed plants than in the plants with the WT *cryIA(b)* gene (Table 2). The modified genes (PM and FM) were routinely expressed above the biological threshold set for insect control in tomato and tobacco. This occurrence was less frequently observed with the WT gene, especially in tobacco.

Immunological analysis of plants expressing either the WT, the PM, or the FM *B.t.k.* insect control protein genes demonstrated that modification of the *B.t.k.* gene resulted in increased insect control protein levels in both tobacco and tomato. The distribution of these plants by the levels of insect control protein found in leaf tissue revealed that the PM and FM genes had a greater percentage of plants with higher levels of detectable protein compared to transgenic plants with the WT gene (Fig. 2). Over 200 transgenic tomato plants with the WT *cryIA(b)* gene were screened; 53 showed insect control and in only 3 plants were the levels of protein detectable by Western blot analysis. Insect control protein was barely detectable in 2 plants, near the limit of detection in this assay (≈1 ng per 50 μg of total protein).

Tomato plants expressing the PM gene showed an increase in the percentage of insecticidal plants (Table 2) and in the levels of insect control protein detected (Fig. 2*A*). The majority of the plants transformed with the PM gene fall into the 1- to 10-ng level and a small percentage (2 of 25 plants) of the plants are in the lower end of the 10- to 30-ng range (0.02%).

Tomato plants that expressed the FM genes contained a similar percentage of insecticidal plants compared to plants with the PM gene (Table 2). A substantial number of plants (10 of 29; 34%) were found to contain high insect control protein levels (Fig. 2*A*). Slightly higher overall levels of insect control protein were seen with tomato plants transgenic with

Table 1. Summary of changes made in the PM and FM *cryIA(b)* genes compared to the WT *cryIA(b)* gene

	WT	PM	FM
No. of bases different from WT	—	62 of 1743	390 of 1845
No. of codons different from WT	—	55 of 579 (9.5%)	356 of 615 (60%)
G+C content	37%	41%	49%
No. of potential PPSS	18	7	1
No. of ATTTA sequences	13	7	0

PPSS, plant polyadenylylation signal sequences.

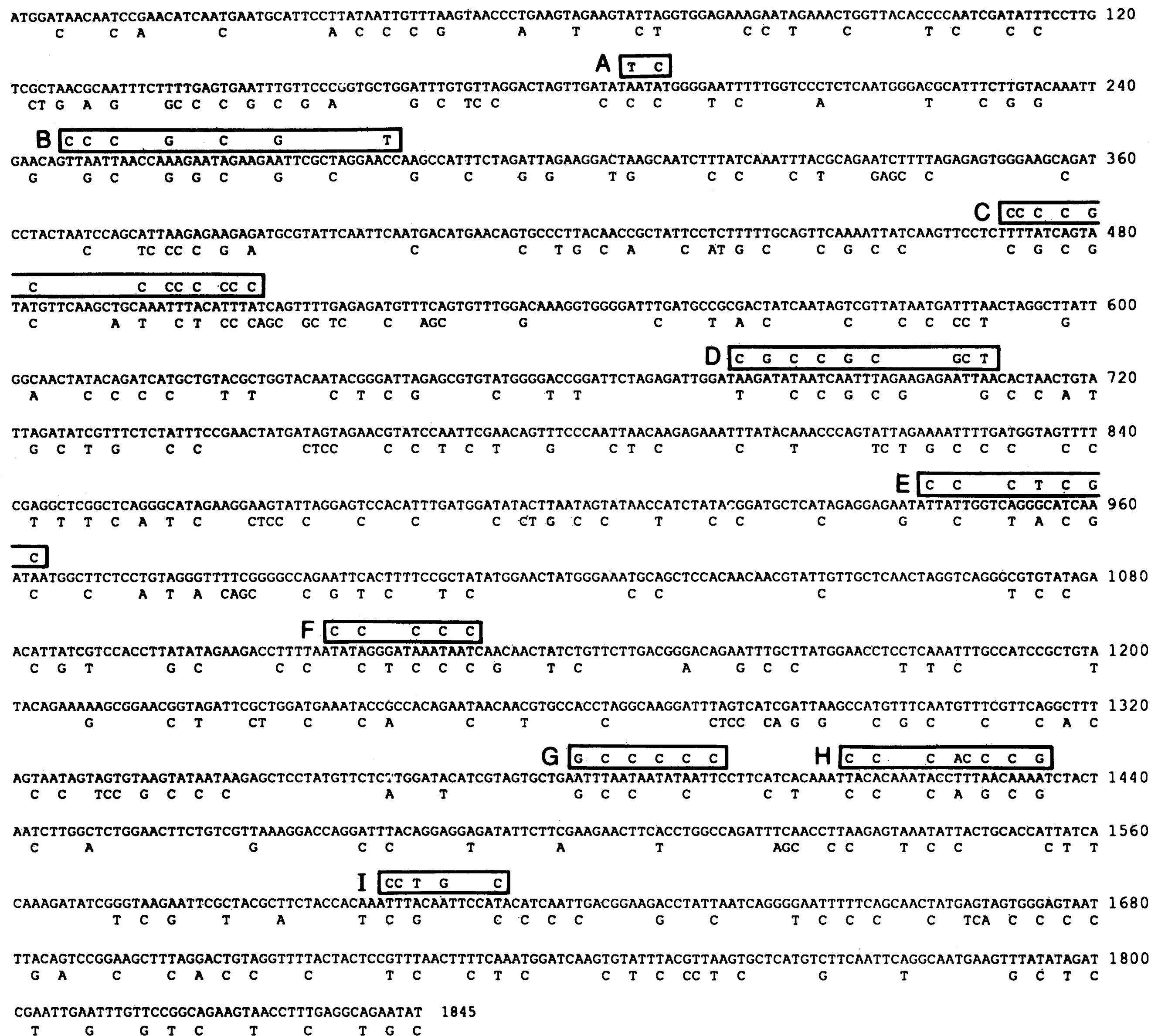

FIG. 1. DNA sequence of the *cryIA(b)* gene on the numbered line with the modifications found in the PM gene on the line above and the FM gene on the line below. The differences between the WT and PM genes are within the labeled boxed areas (A–I).

pMON5383, a truncated *cryIA(c)* FM gene with substantial homology to the *cryIA(b)* FM gene depicted in Fig. 1. Among the 52 FM *cryIA(c)* transgenic tomato plants that were immunologically analyzed, 10 (20%) were at or above the 30- to 100-ng range with several plants approaching 100 ng per 50 μg of total protein. Among the most highly expressing transgenic tomato plants for each insect control protein gene, the FM genes appeared to be expressed 5- to 10-fold higher than the PM gene and up to 100-fold higher than the WT gene.

In tobacco plants, the WT gene was more poorly expressed than in tomato, below the detection limit of the Western blot analysis in the 3 plants showing insect control against THW. Analysis of transgenic tobacco plants (Fig. 2*B*) with the PM and FM genes revealed results similar to those found in tomato. There was an increase in the frequency of plants with insect control (Table 2) and the levels of insect control protein in plants with the PM gene compared to the WT. As in tomatoes, several tobacco plants (2 of 25; 8%) with the PM gene contained 10–30 ng of insect control protein per 50 μg of total plant protein.

Transgenic tobacco plants with the FM gene demonstrated improved expression of the *B.t.k.* protein over the PM gene plants. Several tobacco plants with the FM genes contained detectable levels of insect control protein >50 ng per 50 μg of total protein (>0.1%). Fig. 2*B* shows a high percentage of tobacco plants with the FM *cryIA(b)* gene in the 30- to 100-ng range but only 13 plants with this construct were analyzed. Extensive analysis was done with pMON5390, a FM trun-

Table 2. Comparison of the fraction of plants exhibiting insect control with the WT, PM, and FM genes

Gene	Plants exhibiting insect control/ total kanamycin-selected plants	
	Tobacco	Tomato
WT *cryIA(b)*	3/54 (6%)	53/204 (26%)
PM *cryIA(b)*	25/42 (60%)	40/63 (63%)
FM *cryIA(b)*	12/22 (55%)	32/55 (58%)
FM *cryIA(c)*	—	52/97 (54%)
FM *cryIA(c)**	48/95 (51%)	—

*The coding region of the N-terminal amino acids of this FM *cryIA(c)* gene has been deleted as in the WT and PM *cryIA(b)* genes.

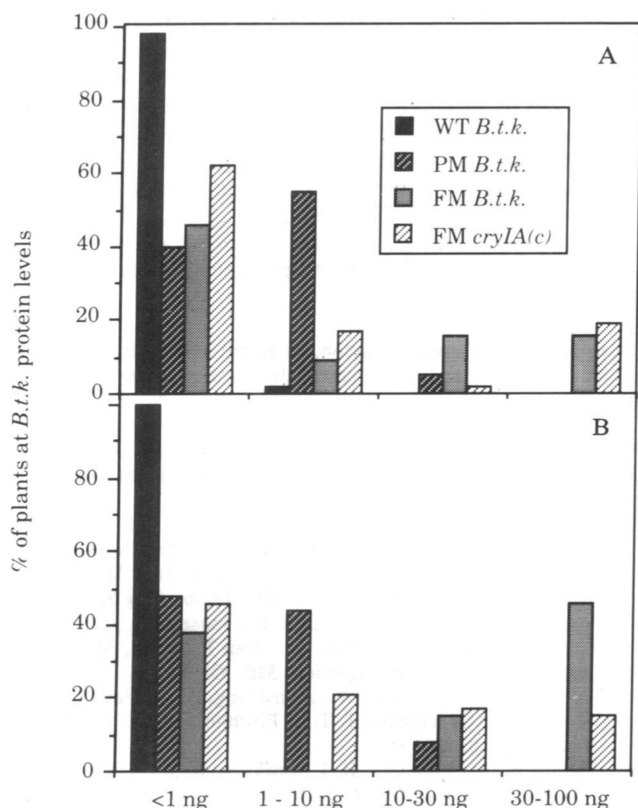

FIG. 2. Distribution of tomato (*A*) and tobacco (*B*) plants that exhibited insect control. The numbers of tomato plants that exhibited insect control used to calculate the percentage of *B.t.k.* protein levels are as follows: WT gene, 53; PM gene, 63; FM gene, 55; FM *cryIA(c)*, 97. The numbers of tobacco plants are as follows: WT gene, 3; PM gene, 42; FM gene, 22; FM *cryIA(c)*, 95. Levels of insect control protein are per 50 μg of total plant protein.

cated *cryIA(c)* gene similar to the *cryIA(c)* gene found in pMON5383 except that the coding sequence for the first 28 amino acids had been deleted as in the WT and PM genes. A high percentage of the plants (50%) that exhibited insect control contained protein at detectable levels. As with the FM gene in tomato, one-third (31%) of the plants were in the 10- to 100-ng range of insect control protein per 50 μg of total protein.

The higher frequency of transgenic plants with the PM and FM genes exhibiting insect control compared to the plants with the WT gene was a good indication that the modifications to the *B.t.k.* coding sequence had improved gene expression. Screening of a large number of plants with the WT gene failed to yield any that produced >1 ng of insect control protein per 50 μg of total protein, while >10% of the plants with the FM genes produced 30–100 ng of insect control protein in both tomato and tobacco. Because of the large numbers of plants analyzed, the overall similarity of the genetic constructs and the large variance in both average and peak expression obtained with the WT, PM, and FM genes, we believe that the data reflected inherent gene expression differences. Gene copy number did not appear to differ for the different coding sequences. The most highly expressing tomato plants containing either the WT, PM, or FM gene were analyzed genetically and the insect control protein gene segregated as a single dominant locus. Highly expressing tobacco and tomato plants with the FM *cryIA(b)* and FM *cryIA(c)* genes were identified that contained insect control protein at levels of 100–150 ng (0.2–0.3%).

mRNA Analysis. The levels of *cryIA(b)*-specific mRNA in plants did increase in plants with the PM gene and the FM gene compared to plants with the WT gene (data not shown). Quantitative S1 nuclease mRNA analysis revealed that selected tomato plants with the PM gene had a 10-fold increase in insect control protein levels and an average 2.5-fold increase in *cryIA(b)* mRNA over tomato plants with the WT gene. Tomato plants with the FM *cryIA(b)*, which exhibited a 50-fold increase in insect control protein levels over tomato plants with the WT gene, had a 5-fold increase of *cryIA(b)* mRNA levels. For two selected plants, levels of mRNA from the plant with the PM gene differed by <20% from that in a plant with the FM gene, yet the plant with the FM gene contained a 4-fold higher level of insect control protein. Northern blot analysis of the best plants with the WT gene and plants with the PM and FM *cryIA(b)* genes indicated the *cryIA(b)* mRNA was of the expected size.

Identification of Important Regions for *B.t.k.* Gene Expression in the PM Gene. The FM gene contains extensive differences compared to the WT gene (Table 1). The PM gene differs from the WT gene by 3% of the nucleotides (Fig. 1), yet these changes account for at least a 10-fold increase in insect control protein levels in tobacco. Plasmids containing a fraction of the total changes (Table 3) were used to obtain transformed, kanamycin-selected tobacco plants that were screened by insect bioassay. *B.t.k.* protein levels comparable to the PM gene (pMON5370) were obtained when the changes in the 5′ half of the coding sequence were present (pMON10707). The gene with the changes in the 3′ half alone (pMON10706) did not differ from the WT gene; there was no immunologically detectable insect control protein. Changes with oligonucleotide B (pMON10537) appeared to be the most significant section to elicit the increased insect control protein levels.

The changes made by oligonucleotide B were not the sole effector of increased levels of insect control protein in plants. A construct that contained a fully modified 5′ one-third of the gene, including oligonucleotide B (pMON5378), resulted in plants with levels of insect control protein comparable to plants with the PM gene. By comparison, replacing the first one-third of the FM gene with the homologous WT section (pMON10538) resulted in lower levels of insect control protein than previously observed for the FM gene. The expression of the hybrid gene of pMON10538 indicated that it is possible to achieve increased *B.t.k.* expression compared to the WT gene without altering the region of oligonucleotide B. These results indicate that a single region is probably not solely involved and that changes throughout the sequence as found in the FM gene were required for the highest levels of expression of the insect control protein gene in plants.

Table 3. Analysis of the contributions of individual regions to increased expression of the *B.t.k.* PM gene in tobacco

Construct	Oligonucleotides	Plants with insect control/plants screened	Protein level, % of total protein
pMON5370	A–I*	23/48 (48%)	0.02–0.03†
pMON10707	A,B,C,D	19/48 (40%)	0.028
pMON10706	E,F,G,H,I	1/53 (2%)	ND‡
pMON10539	A	2/55 (4%)	ND‡
pMON10537	B	17/57 (30%)	0.016
pMON10540	A,B	23/88 (26%)	0.015
pMON10705	C	1/47 (2%)	ND‡
pMON10538	1/3 WT, 2/3 FM	19/62 (30%)	0.016
pMON5378	1/3 FM, 2/3 WT	9/20 (45%)	0.02–0.03†

*The locations of the individual regions (A–I) are depicted in the sequence shown in Fig. 1.
†The levels of CryIA(b) were determined by Western blot analysis.
‡The level of CryIA(b) in these plants was too low to be detected by ELISA or Western blot.

DISCUSSION

The PM and FM genes have dramatically increased insect control protein levels in plants. This appears to be one of the largest increases in gene expression in any system obtained solely through modification of the coding sequence. The rationale for these designs was based on the elimination of sequences such as potential polyadneylylation signal sequences, ATTTA sequences, and A+T-rich regions. The increased expression was generic across several plant species—tobacco, tomato, cotton (5)—and was obtained with two distinct genes, *cryIA(b)* and *cryIA(c)*.

The PM gene contained a subset of the changes found in the fully modified gene, yet produced large increases in insect control protein levels in tobacco and in tomato (at least 10-fold). The lack of extensive changes in the PM gene (3% of the total nucleotides) and the localization of a region critical to increased expression (oligonucleotide B) argues against the hypothesis that the presence of rare plant codons is primarily responsible for the previously reported low level expression of WT insect control protein genes in plants. Analysis of the region of oligonucleotide B revealed no inverted repeats, palindromic sequences, or other apparent secondary structures that distinguished this region from other regions of the structural gene. This region does contain three potential polyadenylylation signal sequences (two AACCAA and one AATTAA). Northern blot analysis of total RNA did not detect truncated *cryIA(b)* mRNA in plants expressing the WT gene. It is possible that the incomplete functioning of a polyadenylylation signal led to processing without polyadenylylation causing instability. Another possibility is that the region of oligonucleotide B contains an as yet undefined regulatory sequence dependent on the context of its surrounding sequence.

The region of oligonucleotide B is not the sole suppressor of insect control protein gene expression in plants. The fully modified C-terminal two-thirds of the truncated insect control protein gene plus an N-terminal WT one-third containing a sequence unchanged in oligonucleotide B (pMON10538) resulted in levels of insect control protein in plants equivalent to the PM gene or ≈10-fold lower than for the FM *cryIA(b)* gene. One interpretation of these results is that the presence of the WT sequence in the 5′ third of the gene, including the region of oligonucleotide B, depresses the expression of the FM gene ≈10-fold. Alternatively, the aggregate of the changes contained in the 3′ two-thirds of the FM gene could partially compensate for the presence of the WT sequences in the 5′ one-third of the gene. These observations suggest that several factors and regions of the gene are involved in the low levels of insect control protein observed with the WT gene in plants. The highest levels of insect control protein that we have observed in plants were obtained with the FM genes, both *cryIA(b)* and *cryIA(c)*.

The major block to efficient WT *cryIA(b)* gene expression in plants appeared to be translational and not at the level of stable accumulation of *cryIA(b)* mRNA. mRNA levels were increased in plants with the PM and FM genes, but the level of mRNA increase was not directly proportional to the level of protein increase in the best of the PM and FM plants. It is not clear that the increased mRNA levels must be directly proportional to the increased protein expression. Analysis of the mRNA levels of some of the PM and FM plants indicated that comparable amounts of FM mRNA led to the production of more insect control protein than PM mRNA, suggesting that the presence of predominantly plant preferred codons in the FM gene has improved its overall translational efficiency.

The impact of the exceptional levels of expression that the PM and FM genes provide is most apparent in the efficacy these plants exhibit to withstand insect attack. The use of these plants in an integrated pest management system will expand the options of farmers to protect their harvest from insect destruction.

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