EXPRESSION OF Bacillus thuringiensis *δ-ENDOTOXIN GENES IN TRANSGENIC PLANTS*

Gustavo A de la Riva[1] and Michael J Adang[2]

[1]*División de Plantas. Centro de Ingeniería Genética y Biotecnología. P.O. Box 6162, C.P. 10600, Ciudad de La Habana, Cuba.*

[2]*Department of Entomology. University of Georgia, Athens, GA 30602, USA.*

REVISIÓN

ABSTRACT

The cloning and expression of *Bacillus thuringiensis* δ-endotoxin genes in transgenic plants have been used with the objective of protecting the crops from insect attack. The increased expression of the insecticidal *cry* genes in plants has been critical for the development of genetically transformed plants with agronomically acceptable levels of insect resistance. Low expression levels of such genes also have an environmental implication: the release of low expressing insect-tolerant transgenic plants may result in the rapid appearance of resistance to the Cry toxin in the target insect. The problem of the expression of *B. thuringiensis cry* genes is due to the expression of bacterial prokaryotic genes in higher plants or in any other eukaryotic organism. Fully modified genes can express up to 100-fold higher levels of the insecticidal toxin compared to those obtained when a wild-type bacterial gene is expressed. We describe the most important aspects present in the bacterial wild-type *cry* genes affecting their expression in transgenic plants. The analysis includes aspects of transcriptional regulation, mRNA stability, preferences in codon usage and translational efficiency. According to these considerations, modified *cry* genes have been reconstructed allowing to increase the expression levels in transgenic plants.

Key words: **plant protection, *cry* genes, modified genes**

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RESUMEN

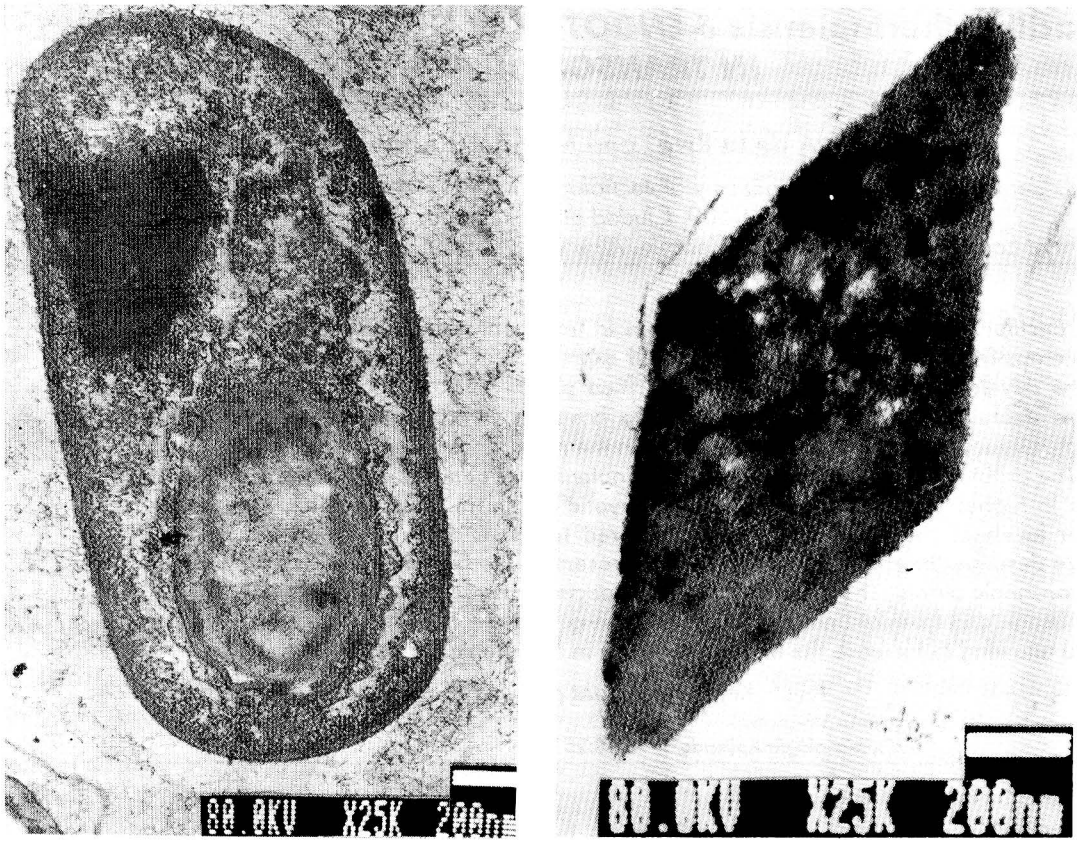
La clonación y expresión de los genes que codifican las δ-endotoxinas de *Bacillus thuringiensis* en plantas transgénicas han sido utilizadas para proteger las cosechas contra el ataque de insectos. La obtención de altos niveles de expresión de los genes insecticidas *cry* ha sido un aspecto crítico para el desarrollo de plantas genéticamente transformadas con niveles agronómicamente aceptables de resistencia a los insectos. Los bajos niveles de expresión de estos genes tienen también una implicación ambiental: la liberación de líneas de plantas transgénicas con bajos niveles de expresión pero tolerantes en determinado grado al ataque de insectos puede resultar en una rápida aparición de insecto-resistencia a la toxina Cry por parte de la especie blanco. El problema de los bajos niveles de toxina radica en que los genes *cry* de *B. thuringiensis*, bacterianos y prokaryotas son expresados en plantas superiores u otros organismos eukaryotas. Genes totalmente modificados de acuerdo a las características de los genes nucleares de plantas han permitido obtener niveles de expresión de hasta 100 veces superiores comparando con sus homólogos salvajes. Nosotros describimos los aspectos más importantes presentes en los genes bacterianos *cry* de *B. thuringiensis* y que afectan su expresión en las plantas transgénicas. Este análisis comprende los aspectos de regulación transcripcional, estabilidad del mRNA, uso de codones y eficiencia traduccional. De acuerdo con estas consideraciones se han reconstruido genes *cry* modificados que permiten aumentar niveles de expresión en plantas transgénicas.

Palabras claves: **protección de planta, genes *cry*, genes modificados**

Introduction

Bacillus thuringiensis is a Gram-positive spore-forming soil bacterium widely used in agriculture as a biological pesticide. The entomocidal activity resides mainly in a parasporal protein inclusion body or crystal that is produced during sporulation (Figure 1). That crystal is composed by one or more δ-endotoxins. Because these toxins combine high potency for target insects with safety for other animals, they are valuable tools for pest insect control. The mode of action is still a matter of research; but in general, *B. thuringiensis* inclusion bodies dissolve in the larval midgut under alkaline conditions and proteolytic cleavage, releasing one or more insecticidal monomers. These monomers bind specifically in the insect midgut brush border to the binding proteins also called receptors. The insertion of the active toxin into the membrane of the midgut epithelial cells disturbs the osmotic balance by generating pores in the cell membrane, leading to cell lysis (1, 2). Lysis of the midgut cells and its perforation lead to paralysis and death of the insect (3). *B. thuringiensis* is capable of disrupting the potassium flux created by proton pumps in the midgut globet cells. Insecticidal specificity is related to the

Corresponding author



A B

Figure 1. *A transmission electron microscope view of sporulating* Bacillus thuringiensis *var. kurstaki HD-1 (A) and bipyramidal crystal parasporal body characteristic for this strain (B).*

specificity of the binding of a toxin to midgut receptors, but the molecular basis of toxicity is the ability to form an ion channel.

Most strains are active against certain larvae of lepidopteran species but other isolates show toxicity against dipteran, coleopteran and nematodes (Figure 2) (4). For more than three decades, formulations of *B. thuringiensis* have been used as biological pesticides to control agricultural pest and insect vectors of a variety of human and animal diseases. δ-endotoxins are encoded by *cry* genes which have been extensively studied and expressed in a wide number of prokaryotic organisms (*Escherichia coli, Bacillus subtilis, Pseudomonas fluorescens*) and plants. The transfer and expression of such genes in plants has provided a potentially powerful alternative for the protection of crops against insect damage. The expression of bacterial genes such as the genes *cry* implies the use of plant regulatory sequences as well as the modification of the gene's coding region according to the characteristics present in other plant genes.

We present some of the most important aspects involved in the expression of bacterial genes in transgenic plants. Particular emphasis was made in the structure and composition of the coding region of *B. thuringiensis cry* genes and their decisive role in the low yields of the recombinant proteins when they are expressed in transformed plants.

Plant Genetic Engineering

It has become possible to introduce and express foreign genes in plant cells. The first record on transgenic tobacco plants expressing foreign genes goes back to the past decade (5, 6). Since that outstanding moment in plant sciences, a great progress has been obtained in tissue culture, plant transformation and plant molecular biology. At first, most genetically engineered plants were transformed by *Agrobacterium*-mediated transformations (6). *Agrobacterium tumefaciens*, a plant pathogen causing tumorous crowngalls on infected dicotyledonous plants, does not infect monocotyledonous plants; for this reason, many economically important plants such as cereals, remained inaccessible for genetic engineering manipulation for a long time. For those cases alternative direct transformation methods have been developed (7), such as polyethyleneglycol-mediated transfer (8), microinjection (9), protoplast and intact cell electroporation (10-14) and particle bombardment or gene gun technology (15). The gene gun technology and the electroporation of

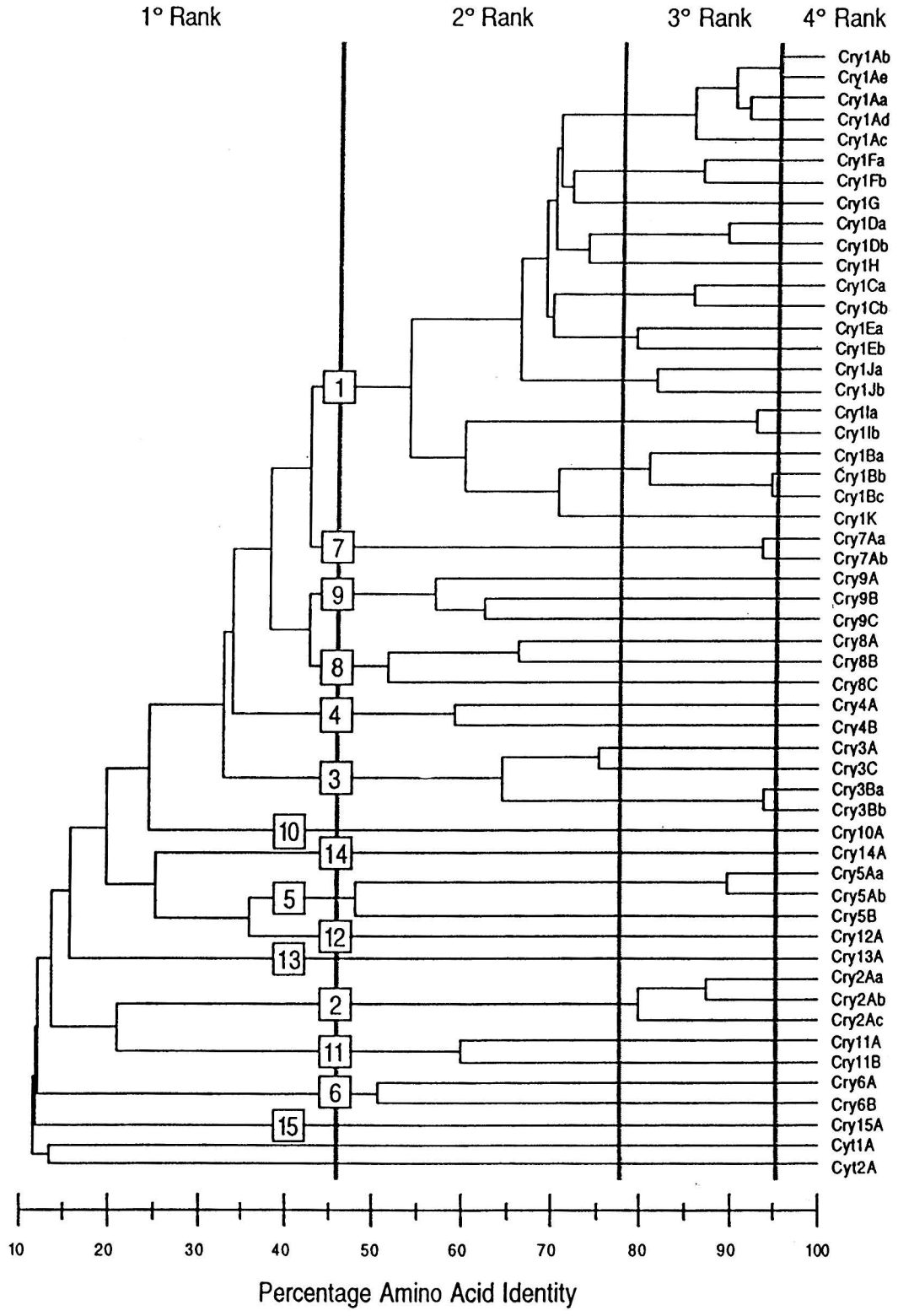


Figure 2. *Possible evolutionary relationships between* Bacillus thuringiensis *δ-endotoxins. The Fitch-Margoliash algorithm was used originally to compare the aminoacid sequences of 54 active toxin regions (Internet, home page* B. thuringiensis *http://www.susx.ac.uk/ and users/bafnG/bt/index.html).*

intact cells allow the direct delivery of DNA into plant cells obviating the difficulties in the regeneration of transgenic plants from transformed protoplasts. However, significant parts of the regenerated plants are not wholly transformed but form mosaic transgenic plants. To avoid this problem, the development of high efficiency *Agrobacterium*-mediated transformations of monocotyledonous plants is necessary. Some results in *Agrobacterium*-mediated transformations of monocotyledonous plants have been reported (16-19) and the most important groups increase the efforts in this direction.

The generation of transgenic plants with resistance or tolerance to insect and pest attack is one of the most extensively explored fields in plant genetic engineering. The advantages of the biodegradability, selectivity and total safety for man and the environment have led to the use of BT-based products. Despite the distinct advantages of BT-based insecticides, certain reasons limit their use. These include ineffective field performance, mainly because of the toxin susceptibility to the environmental conditions and ultraviolet radiation from sunlight and, in some cases, the difficulty of the toxin for reaching the target pest inside the stem or leaves (stem borer, leave mining). Recombinant DNA technology has the potential to complement the efforts of plant breeders. The cloning and expression of *cry* genes in *E. coli* (19-24) and *B. subtilis* (22, 25) allow the study of the structure, molecular organization of individual *cry* genes and the entomocidal activity of the individual recombinant δ-endotoxins. Transgenic plants that are resistant or tolerant to insect attacks have been produced in tomato, tobacco, potato, cotton (26-32), maize (33) and more recently in sugarcane (34).

Expression of Bacterial Genes in Plants

During the past decade a great number of different plant genes have been cloned and well characterized. According to the accumulated data, we can conclude that the plant genes are very much like animal or yeast genes. The plant genes use the same genetic code (are split by introns) and basically use the same approaches of gene regulation. However, plant genes have differences compared to animal genes. In plant genes the requirements for splice site recognition are subtly different. Differences can be observed in plant promoters; although in general, the basic aspects remain the same. However, they are extremely different when they are compared to bacterial genes. This aspect is very important to understand why the expression levels of bacterial genes expressed in plants is very low.

Reports on engineering plants with different crystal protein genes, mostly under the control of the strong and constitutive CaMV 35S promoter, have been published. Compared to other genes transferred to plants, the *cry* genes are weakly expressed in transgenic plants. Plants transformed with the full length *cry* sequences encoding the protoxin have extremely low levels of δ-endotoxin. Significant increases on recombinant protein production are obtained when a truncated version, encoding only the toxic fragment of the protoxin, is transferred and expressed in the plant. Using a truncated version of the *cry* IA(b) gene, the recombinant toxin detected in the leaves of transgenic tobacco plants represented up to 0.07 % of the total leaf protein; while only 0.0001 % is reported when full length genes were expressed (26). Even when only truncated versions of *cry* genes are considered for the generation of transgenic plants, the expression level remains low. Despite this fact, plants showed a remarkable entomocidal activity against target insects (27, 34). Although low expressing plants were protected against certain pests, higher expression levels and more specific crystal proteins are required to control agronomically important insects.

Plant Promoters

The detailed studies clearly demonstrated that the promoter is not simple at all but a rather complex array of various regulatory *cis*-acting elements and the sum of all these specialized "promoter units" that results in a constitutive or specialized promoter. This model is also applicable to other eukaryotic regulatory systems and it is defined as the combinatorial model of promoter function (35). Small *cis*-acting elements have usually been localized in the upstream sequences that can confer correct regulation upon the foreign gene. Proteins that bind these specific *cis*-acting elements have been isolated and their coding genes cloned and characterized. Now, one of the most important goals in plant molecular biology is to understand how the different transcription factors cooperate with each other and interact with the *cis*-acting elements in promoters, bringing the fine way of regulation of individual genes.

The high yields of production of foreign proteins in transgenic plants by efficient gene expression re-

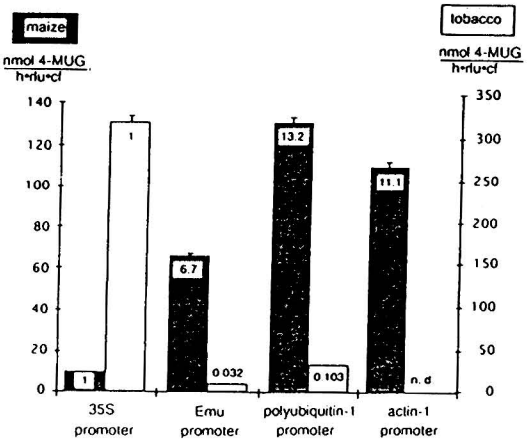


Figure 3. *Relative corrected GUS activities of four widely used promoter constructs expressed in cell suspensions of maize and tobacco after biolistic transfer. The relative promoter strength is given as a value in the upper part of the bars and it is based on the CaMV 35S promoter to which the value 1 was assigned (36).*

mains as one of the most important aspects in plant molecular biology. The 35S Cauliflower Mosaic Virus (CaMV) is widely used in plant genetic engineering and it is considered as one of the strongest promoters available up to now. However, the 35S CaMV promoter shows preferential activity in dicotyledonous plant cells (Figure 3) (36). The base composition and function of 35S CaMV promoter sequence has been established (35, 37). Better results are obtained in monocotyledonous plants by using other strong and constitutive promoters: the rice actin 1 promoter (38), the maize *Emu* promoter (39) and the maize polyubiquitin 1 promoter (40, 41). The combination of these elements, previously reported as important for transcriptional activity in their original gene systems, offers the possibility to obtain promoters that could drive gene expression more efficiently or in a specific manner. In the design of chimeric promoters for the high efficiency expression of foreign genes in dicotyledonous or monocotyledonous transgenic plants, elements from different gene systems are often combined. This includes small enhancer sequences such as the 18 bp enhance element of the *A. tumefaciens* octopine synthase promoter (18 bp OCS). Usually, such elements are chemically synthesized and inserted in the promoter sequence in single or multiple forms at different distances from the gene transcription start.

Expression of Bacterial Genes in Transgenic Plants. Modification of *Cry* Genes

Modifications of the *cry* coding sequence resulted in increased expression levels in tobacco, tomato cotton, potato (30-32) and maize (33). To make the bacterial *cry* genes more "plant-like", it is necessary to analyze some important aspects of the gene expression process in order to determine the modifications in the typical *cry* gene sequence that could enhance the gene expression in plant cells. The modifications we suggest are:

A- Introduction of the preferential codon usage presented by plant genes.

B- Elimination of CG and TA dinucleotides at codon positions 2 and 3.

C- Conservation of the A+T base composition according to the proportion presented by plant genes.

D- Modification of sequences that might cause mRNA destabilization, including premature polyadenilation signals, mRNA degradation signals, RNA-polymerase II termination signals and signals for splicing

E- Reduction of secondary structure hairpins of the mRNA.

F- Optimization of the ATG consensus flanking nucleotides for protein translation initiation and termination.

G- Introduction of viral untranslatable mRNA leader to improve the initial steps of translation.

The codon usage patterns in bacteria and plants exhibit remarkable differences (Table 1) (42). The modified gene could contain less AT-rich regions but it is recommended that its codon usage pattern must be intermediate between the more AT-rich bacterial *cry* codons and the more GC-rich monocotyledonous codon preference (29). The dinucleotide frequencies of GC used at codon position 2 and 3 must be conserved when no contradictions with plant preferential codon usage are observed. The CG dinucleotides are strongly avoided in plant genes, probably due to regulation involving methylation (42). Codons for Ala, Pro, Ser and Thr containing CG at positions 2 and 3 are rarely used in plants. In dicotyledonous plants the CG-ending codons are always the least favored but in monocotyledonous plants this is not always the case. In most eukaryotes, including plants, the TA-ending codons are also less favored. Concerning the A+T composition, the *cry* genes show about 60 % to 70 % A+T, representing a proportion 10 % to 15 % higher than that found in typical plant gene coding regions. In plants the A+T is particularly abundant in the intergenic and regulatory regions.

In eukaryotic cells, as a result of transcription, primary transcripts from nuclear genes called heterologous nuclear mRNA (hnRNA) are generated and then extensively processed. The hnRNA processing includes 5' capping, intron splicing and polyadenilation to form the mature polyA-mRNA. This is the only translatable form of mRNA. Control of gene expression at the level of mRNA stability is very important in plants (43, 44). Plants are sessile organisms, unable to move from adverse situations and forced to respond to environmental stimuli by altering endogenous gene expression. An important aspect of this regulation is the control of transcript processing. Most of RNAs in higher eukaryotes are relatively stable with a half-life period in the order of several hours (43). For many transcripts, differences in mRNA stability contribute to the establishment of steady-state mRNA levels and also to the speed at which those levels are achieved after a modification in transcription rate (45). Proteins that are required only transiently by the cell are encoded by unstable transcripts with half-lives in the order of an hour or less. Unstable transcripts are expected to act mostly in the rapid modification in gene expression that are present at mRNA level. This is necessary because, even after the immediate stop of transcription, such transcripts could remain functional for translation during several hours (46). In plants, the genes with a functional role in growth, development and rapid environmental responses are known to be encoded by unstable transcripts (46-48). One example is the phytocrome (*Phy A*)

Table 1. *Codon usage in pooled sequences of* Bacillus thuringiensis *cry genes and nuclear genes of higher plants. The analysis included 30 410, 46 547, 39 155 and 39 155 codons from* B. thuringiensis, *dicotyledonous, and monocotyledonous plant genes respectively.*

Amino Acid	Codons	*Bacillus thuringiensis* n= 40		Plants n= 280		Dicotyledonous Plants n= 155		Monocotyledonous Plants n= 125	
		No.	%	No.	%	No.	%	No.	%
Gly	GGG	356	19.4	1157	17.1	391	11.0	764	24.0
	GGA	784	42.8	1980	29.3	1448	40.6	533	16.7
	GGT	480	26.2	1654	24.5	1183	33.1	478	15.0
	GGC	213	11.6	1963	29.1	1545	15.3	1418	47.3
Glu	GAG	1475	74.1	2777	58.5	1471	48.2	1304	77.1
	GAA	517	25.9	1971	41.5	1583	51.8	388	22.9
Asp	GAT	1338	81.6	1740	45.9	1383	60.4	356	23.8
	GAC	301	18.4	2048	54.1	908	39.6	1139	76.2
Val	GTG	328	16.6	1911	33.3	913	28.2	1006	40.1
	GTA	824	41.6	540	9.4	378	11.7	165	6.6
	GTT	605	30.9	1851	32.2	1406	43.4	439	17.5
	GTC	216	10.9	1440	27.1	540	16.7	897	35.8
Ala	GCG	255	15.3	1170	16.5	913	28.2	940	26.5
	GCA	623	37.4	1457	21.0	378	11.7	513	14.5
	GCT	563	33.8	2383	33.5	1406	43.4	736	20.7
	GCC	225	13.5	2083	29.0	540	16.7	1359	38.3
Arg	AGG	146	9.3	943	25.7	503	24.5	439	27.9
	AGA	666	42.4	380	21.3	638	30.5	137	8.7
	CGG	61	3.9	343	9.3	70	3.4	270	17.2
	CGA	262	16.7	266	7.3	192	9.2	71	4.6
	CGT	353	22.5	592	16.0	461	22.0	133	8.5
	CGC	81	5.2	746	20.4	228	10.4	521	33.2
Ser	AGT	366	25.2	711	12.3	564	16.9	153	6.3
	AGC	185	8.2	1208	20.9	592	17.8	618	25.3
	TCG	149	6.6	557	9.6	168	5.0	388	15.9
	TCA	484	21.6	1011	17.5	671	20.1	337	13.8
	TCT	623	27.8	1195	20.7	829	24.9	360	14.7
	TCC	237	10.6	1097	19.0	508	15.3	587	24.0
Lys	AAG	243	18.3	2957	67.8	1665	59.2	1292	83.1
	AAA	1085	81.7	1405	32.2	1145	40.8	262	16.9
Asn	AAT	1657	74.9	1294	37.9	1025	47.8	270	21.3
	AAC	557	25.1	2117	62.1	1118	52.2	998	78.7
Met	ATG	456	100.0	1988	100.0	1125	100.0	865	100.0
Ile	ATA	600	30.1	643	15.4	447	17.8	199	12.0
	ATT	1040	52.3	1577	37.9	1178	46.7	403	24.4
	ATC	350	17.6	1945	46.8	894	35.5	1053	63.6
Thr	ACG	381	17.9	506	12.1	154	5.2	352	20.9
	ACA	894	40.8	960	22.9	1171	39.8	227	13.5
	ACT	603	28.5	1303	31.1	983	33.4	321	19.0
	ACC	271	12.8	1423	33.9	638	21.6	787	46.0
Trp	TGG	429	100.0	1080	100.0	601	100.0	485	100.0
Cys	TGT	213	70.5	574	34.7	382	45.8	188	23.1
	TGC	89	29.5	1080	65.3	452	54.2	626	76.4
Tyr	TAT	1171	79.5	926	33.9	652	43.2	274	22.4
	TAC	302	20.5	1808	66.1	857	56.8	951	77.6
Leu	TTG	289	10.9	1534	21.4	1025	26.7	509	15.2
	TTA	1244	47.1	523	7.3	410	10.7	110	3.3
	CTG	153	5.8	1208	16.8	294	7.7	916	27.4
	CTA	332	12.6	634	8.9	303	7.9	337	10.1
	CTT	511	19.4	1542	21.5	1108	28.9	439	13.1
	CTC	110	4.2	1731	24.1	694	18.1	1034	30.0
Phe	TTT	1047	78.9	1362	39.3	955	46.9	411	28.4
	TTC	280	21.1	2108	60.7	1080	53.1	1034	71.6
Gln	CAG	232	16.9	2468	40.4	699	39.8	1774	40.8
	CAA	1143	83.1	3634	59.6	1057	60.2	2576	50.2
His	CAT	499	85.7	745	44.4	475	55.2	266	33.2
	CAC	83	14.3	934	55.6	386	44.8	536	66.8
Pro	CCG	204	16.7	934	16.8	204	7.8	732	24.6
	CCA	557	45.5	2374	42.6	1136	43.6	1237	41.6
	CCT	405	33.1	1311	23.6	885	34.1	427	14.3
	CCC	57	4.7	951	17.0	377	14.5	580	19.5
STOP	TGA	6	15.0	100	35.7	42	27.1	68	54.4
	TAG	6	15.0	78	27.9	28	18.1	32	25.6
	TAA	28	70.0	102	36.4	85	54.8	25	20.0

n= The number of DNA sequences in the sample.
No.= The number of ocurrences of a given codon in the sample.
%= Percent ocurrence for each codon within a given aminoacid in the sample.

message in oats (49). Treatment of etiolated oat seedlings with red light causes a large decrease in the transcription rate of the *Phy A* gene and a rapid decrease in the level of steady-state *Phy A* mRNA.

The *cry* genes contain DNA sequences that might potentially contribute to RNA instability in plants. Some of them are plant polyadenilation signals such as AATAAA and its variants, others could be a potential termination signal for the RNA-polymerase II. The consensus sequences on this type of signal are not completely defined; however, according to results obtained in electroporated *Arabidopsis thaliana* protoplasts, the sequences $CAN_{7-9}AGTNNA$ next to the 3' end of the U2 snRNA coding region is likely to play an important function in transcription termination (50).

There are also mRNA degradation signals such as poly ATTTA (51) and sequences signaling wrong mRNA splicing. Recent results show several different mechanisms of mRNA degradation in eukaryotic cells. One mRNA decay pathway starts by the shortening of the poly(A) tail followed by decapping and 5' to 3' exonucleolytic degradation of the transcript. Some specific transcripts can be degradated through the deadenilation-independent decapping followed by 5' to 3' degradation. This process can also begin by endonucleolytic cleavage in the transcript. All these mechanisms are more or less related and suggest a model of RNA turnover in which polydenilated RNAs are degradated by the "default" pathway initiated by poly(A) shortening (51). The eukaryotic mRNA can be degradated by endonucleolytic cleavage prior to deadenilation (52, 53). The degradation signal ATTTA (AUUUA) can destabilize transcripts in plants as well as in animals. This fact suggests that the pathway of such degradation may be highly conserved among many eukaryotes (53-55). For transcription termination, it is determined that TAA is preferentially used in dicotyledonous plants where monocotyledonous plants prefer the UGA termination codon (Table 1). The most important aspect in the stop codon context is the preference for A in the position +1 the based placed, following the stop codon and the avoidance of C in the same position. It has been demonstrated in *E. coli* that release factors require a tetranucleotide sequence for recognition and the same was observed in rabbit reticulocyte cell-free. Recent data suggest that the plant stop codon context may be UAAA, UGAA and UAGA (56).

The BT-toxin transcripts are generally AU-rich and contain multiple copies of both AUUUA and AUUAA motifs that may contribute to mRNA instability (57). The *cry* IA(b) gene, for example, contains in the coding region 18 potential plant polyadenilation signals and 13 mRNA degradation signals (33). The expression of *cry* genes in transgenic plants requires mRNA levels buffering against modifications in transcription rates and must be stable. For this reasons the sequences must be modified and the signaling consensus eliminated (1, 29) and the consensus sequence of plant translational initiation and plant stop codon context introduced.

The secondary structure hairpins of the mRNA could be reduced in order to enhance the translocation speed of ribosomes through the mRNA (58-61). It has been demonstrated that the negative effect of minor arginine codons on gene expression is suppressed by the introduction of the preferential codons within the first 25 genes in *E. coli* (62, 63). The *cry* IA(b) gene was modified at its 5' end to prevent such structures in the mRNA during initiation of translation which is considered to be one of the critical steps in the gene expression process. The modification consists of the substitution of the first 55 codons by their most abundant homologues in plant genes (Figure 4). The modifications introduced in the coding region cover only 8 % of the full-length transcript. The secondary structure hairpins were predicted for mRNA of wild-type and modified versions of the truncated *cry* IA(b) taking into account the use of the CaMV 35S promoter, the 70 bp TMV Ω-fragment (Figure 4). Increases of up to five folds were obtained when the modified *cry* IA(b) gene was expressed, driven by the CaMV 35S promoter in electroporated tobacco protoplasts (de la Riva, unpublished). Surprisingly, the expression of a modified gene in *E. coli* resulted in over-production of the recombinant toxin although the modifications introduced were designed to enhance the expression in plant cells. These facts suggest a prevailing role of the 5' end secondary structure of mRNA in the modulation of gene expression particularly during the initial steps of translation.

Translation Efficiency

Almost all plant mRNAs are polyadenilated. It has been established that poly(A) increases message stability and it also functions as a regulator of translation efficiency (64). The regulation of mRNA stability is independent from a similar function of the cap at the 5' end. Despite this fact, the poly(A) tail functions as a translation regulator only when the transcript is capped. During initiation, the activity of the cap depends on the poly(A) tail. This synergism suggests that this functional conjunction with the associated protein complex is in communication during translation. This contact could direct protein-protein contacts between eIF-4F and poly(A) binding protein (PAB), or it could be mediated by other factors (65, 66). Translation in plants is more dependent on the factors mentioned above than it is in animals. Little is known about the mechanism involved in the protection from nucleases of mRNA mediated by the poly(A) tail. Coincidentially, the removal of the poly(A) tail may constitute an early step in the mRNA degradation process (51).

In order to form the consensus of the plant translation start sequence, the modification of the ATG flanking sequence consists of the introduction of minimal modifications in the original *cry* sequence but it enhances significantly the yields of the recombinant protein (65). Together with the modification of the coding region, it is common to find the use of viral untranslatable leaders, like the 70 bp TMV Ω-fragment and the 600 bp leader preceding the CaMV open reading frame VII, in order to increase the affinity of mRNA to the small 40S ribosomal subunit (66, 67). During the first step of viral infection, this type of sequences helps viral transcripts to be more "competitive" in binding ribosomes than the host mRNA, increasing the translation efficiency.

The same approach can be designed to favor the termination step of mRNA translation. These elements interact with translation initiation factors present in dicotyledonous plants such as plant-eIF-4E, the cap-binding protein and plant-eIF-4A (a putative RNA helicase). This RNA helicase is thought to unwind secondary RNA structure in the 5' leader of mRNA to enable the scanning ribosome to reach the initiator AUG. The plant-eIF-4A is active in other organisms like *Xenopus* oocytes where they can activate dormant mRNAs. The translation initiation factors from wheat are active in rabbit reticulocyte cell-free. They are encoded by a multigene family of highly divergent genes. The translation initiation factors in dicotyledonous plants enhance the translation of the mRNA carrying the TMV Ω-fragment and interacting with the leader sequence, while in monocotyledonous plants, the recognition of a leader sequence by the translation initiation factor is less effective and in consequence, the enhancement of translation is less favored. On this basis it is possible to understand why the TMV Ω-fragment shows a better effect in dicotyledonous plants (68). None of the mutations in these sequences influence steady-state mRNA level. This fact allows to conclude that the effects must be at a translation level. A translational enhancer derived from tobacco mosaic virus is functionally equivalent to a Shine-Delgarno sequence (68, 69). Such elements have been described for the 5'-leaders of several plant viruses. The elucidation of the function of the viral tRNA-like structure in the cytoplasmatic gene regulation could bring about new ideas for further strategies to improve the expression of foreign genes in transgenic plants (70).

Ribosome translocation was thought to proceed at a constant speed but it has been observed *in vitro* that the ribosome can pause and stack (58). As it was discussed above, we can conclude that the stable hairpin secondary structure mRNA might result in a less efficient translation initiation. The same effect occurs during the elongation of a polypeptide chain and it might also cause pausing and stacking of the ribosomes. Little is known about how elongation is really regulated. It has been established that *in vitro* ribosome translocation is sensitive to pH, which is an important fact for the control of translation during conditions of hypoxia in which the cytosol acidifies (64).

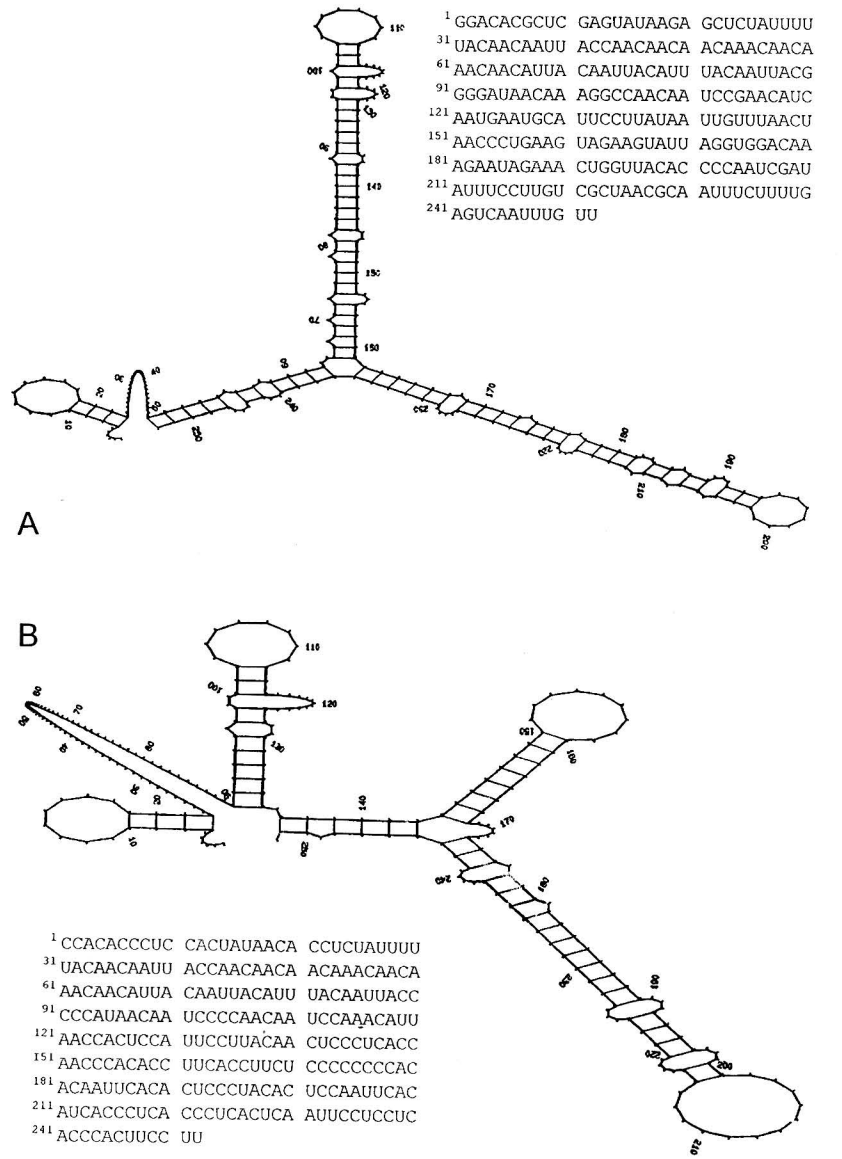


Figure 4. Predicted hairpin structure of mRNA from typical cry IA(c) (A) and modified cry IA(c') (B) genes. The secondary structure of typical mRNA (A) is less favorable for initial steps of translation, including the association of ribosomal 40S and 60S subunits to form the 80S ribosome. The ribosomes translocate through the mRNA in order to reach the initial AUG at position 100 of the transcription start. The hairpin structure of a typical transcript contains 98 hydrogen bonds which represent -11,706 cal/Mol; the modified transcript (B) only 34 which represent -3,822 cal/Mol. The mRNA secondary structures were predicted using the program SQUIGGLES 6,2 (Genetic Computer Group, University of Wisconsin, USA).

Concluding Remarks

The first results on insect control by transgenic plants expressing the *cry* gene were published in 1987 (24, 26). Other experiments have been per-

formed since that moment. The insecticidal activity on tobacco plants were tested in tobacco hornworm (*Manduca sexta*) and tobacco budworm (*Heliothis virescens*), the major pests of this crop. Experiments were also carried out with transgenic potato plants expressing a coleopteran-active *cry* IIIA gene from *B. thuringiensis* var. *tenebrionis*. Transgenic plants resistant or tolerant to insect attacks have been produced in tomato, tobacco, potato, cotton, maize and more recently in sugarcane.

The potentials and the limitations of engineering insect resistance in plants by the expression of *cry* genes have been intensively explored. Plants frequently attacked by insects highly susceptible to *B. thuringiensis* δ-endotoxin can be protected using genetic engineering. The effectiveness of the control depends on the yields of recombinant toxin in the plant. High gene expression level can be obtained only if a modified "eukaryotic-like" *cry* gene is transferred to plants under the control of an efficient promoter. The possibilities for the development of resistance to *B. thuringiensis* δ-endotoxin in genetically modified plants is a polemic topic subject to active and permanent discussion. The fact that different *B. thuringiensis* δ-endotoxins can bind different proteins could help prevent the development of resistance. In laboratory conditions, the development of resistance to one δ-endotoxin can be studied. Therefore; the susceptibility of the resistant insect to others can be known. These tests lead us to suggest the combination of different toxin genes in plants or the alternation of transgenic plants expressing different toxins. Another strategy consists of the limitation of gene expression only to the economically important organs of the plant but this requires an extensive work for the construction and use of efficient and specific tissue promoters. The determination of the molecular aspects of the mode of action of δ-endotoxins, including the structure and function of a larval midgut receptor, will be essential in order to design strategies to control the development of resistance in insects.

During the laboratory evaluation, the most promising transgenic plant lines must be selected for further experiments in field conditions. At the same time, a great number of independent clones is needed in order to obtain some high expressing plant lines. To test all these plants, whenever possible, it is recommended to carry out a challenge experiment, feeding neonate larvae of the target insect on transgenic clones. The selected clones must be characterized by molecular biology (CPR, Southern blot, Northern blot) and immunochemical methods (ELISA, IRMA, Western blot) and the transgenic plant lines showing effective protection against insect attack must be evaluated at field conditions.

The screening projects could lead to the discovery of *B. thuringiensis* strains with novel entomocidal specificities which may be the source of novel δ-endotoxin genes potentially suitable for expression in transgenic plants. Extensive screening programs are being carried out by various groups in order to search for *B. thuringiensis* with such new insecticidal spectra. The production of transgenic plant lines resistant or tolerant to insect attack is not the answer for all insect problems in agriculture but it is an alternative method to be integrated in the current pest management strategies. Other methods for the manipulation and use of *B. thuringiensis cry* genes, including the development of genetically engineered strains with improved -endotoxin production and novel entomocidal specificities are very promising.

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