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Analysis of unstable RNA transcripts of insecticidal crystal protein genes of *Bacillus thuringiensis* in transgenic plants and electroporated protoplasts

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Received 19 October 1991; accepted in revised form 13 February 1991

Key words: Bacillus thuringiensis, cry genes, insecticidal crystal protein, protoplast electroporation, RNA stability, transgenic tobacco

Abstract

We have examined expression of several insecticidal crystal protein (ICP) genes of *Bacillus thuringiensis* in transgenic tobacco plants and electroporated carrot protoplasts. We determined that low levels of lepidopteran toxin *cryIA(b)* ICP gene expression in plants and electroporated carrot cells is due to RNA instability. We used a series of 3′ deleted *cryIA(b)* constructs directed by the cauliflower mosaic virus 35S promoter to demonstrate that this instability is minimally contained in the first 579 bases of the gene in both systems. This instability may result from 5′ → 3′ as well as 3′ → 5′ RNA metabolism. The coleopteran toxic *cryIIIA* gene was also examined in electroporated carrot cells, and found to be poorly expressed. A model for improvement of ICP RNA stability in plants is presented.

Introduction

Bacillus thuringiensis (*B.t.*), a Gram-positive soil bacterium, produces crystalline protein inclusions during sporulation that are specifically toxic to lepidopteran, dipteran and coleopteran insects. The crystal inclusions contain one or more proteins with subunit M_r of 27–160 kDa [22, 61]. Most lepidopteran-active insecticidal crystal proteins (ICPs) are protoxins of M_r 130–160 kDa that are, upon ingestion, proteolytically cleaved in the insect midgut into smaller, active forms (M_r 60–70 kDa) derived from the N-terminal half of the protein.

Over 42 ICP genes (also called *cry* genes) have been sequenced thus far [61]. Due to their extensive homology, these genes have been subdivided into 13 *cry* gene types [61]. These can be further grouped into four classes (*cryI–cryIV*) based on protein structure and biological host range of the encoded proteins. Our group and several others have reported on the generation of transgenic tobacco and tomato plants using a number of lepidopteran toxic *cryI* genes. Analyses of the

expression of intact or truncated ICP genes in these plants have shown extremely low levels of protoxin or toxin and their RNA transcripts.

We have reported the introduction of intact *cryIA(c)* gene into tobacco plants under the control of the mannopine synthase promoter [1]. Levels of ICP detected by ELISA were very low, and on northern blots, only a 1.7 kb truncated, polyadenylated transcript was observed. Steady-state levels of this truncated RNA were 100-fold lower than RNA levels from the flanking octopine synthase (OCS) reporter gene and neomycin phosphotransferase (NPTII) selectable marker in the same tobacco plants. We proposed that sequences within the coding region of the ICP gene could function as regulatory signals for transcription termination or cleavage/polyadenylation, accounting for these truncated, polyadenylated transcripts.

Barton *et al.* [6] introduced a toxic, truncated *cryIA(a)* gene into tobacco plants under the control of the cauliflower mosaic virus (CaMV) 35S promoter. They showed that on northern blots, most hybridization was to distinct, shorter polyadenylated transcripts, although the predicted full-length RNA appeared as a minor species. They also reported low levels of toxin RNA relative to the adjacent NPTII gene. They suggested that ICP mRNA was unstable due to inefficient post-transcriptional processing or rapid turnover of the message.

Transgenic tomato plants containing a truncated *cryIA(b)* gene under the control of the CaMV 35S promoter were analyzed by Fischhoff *et al.* [14]. They reported low levels of ICP mRNA accumulation in plants, with polyadenylated transcripts of the predicted length detected in callus tissue from transgenic plants. *CryIA(b)* protein levels were too low to quantitate by immunoassay, although plants were reported to tolerate tobacco hornworm (*Manduca sexta*) in laboratory and field tests. These tomato plants, and others containing the *cryIA(b)* under the control of the mannopine synthase promoter [43] in a different tomato cultivar, were reported to tolerate tobacco hornworm, tomato fruitworm (*Heliothis zea*), and tomato pinworm (*Keiferia lycopersicella*) although *cry* protein was immunologically undetectable. The authors concluded that higher levels of expression would be required to control agronomically important lepidopteran pests.

Finally, Vaeck *et al.* [58] generated transgenic tobacco plants containing intact and truncated *cryIA(b)* gene constructs, as well as truncated *cryIA(b)*-NPTII fusions under the control of the mannopine synthase promoter. Very low levels of ICP were detected by ELISA assay in plants containing the intact gene. Plants containing truncated or fusion constructs contained higher levels of protein, but the steady-state level of *cry* gene mRNA could not be reliably detected on northern blots. Ribonuclease protection experiments using the first 186 bases of the ICP coding sequence as a probe indicated that RNA levels were 10-fold lower than protein levels in these plants.

Transgenic plants generated by all four groups were tested for insecticidal activity using feeding assays with *Manduca sexta* neonatal larvae. Although, in most cases, ICP expression correlated with toxicity, Barton *et al.* [6], Fischhoff [14] and M. Adang (pers. comm.) showed some transformed plants were resistant to *M. sexta* even when ICP levels were below detectable limits. The leaves of *Nicotiana* species have been reported to contain naturally occurring substances with toxic effects on *M. sexta* [39].

This study reports data indicating that the low level of ICP gene expression in plants and electroporated carrot cells is due to RNA instability. Using a series of 3′ deleted *cryIA(b)* constructs directed by the CaMV 35S promoter, we demonstrate that some of this instability is retained in deletions containing the first 570 bases of the gene in both transgenic plants and carrot cells. We also present evidence that the truncated, polyadenylated RNA is due to 5′ to 3′ degradation of the ICP mRNA. The coleopteran toxic *cryIIIA* gene was also electroporated into carrot cells and proved unstable. Using the available data we present a model to account for ICP RNA instability in plants.

Materials and methods

DNA constructions

The full-length *cryIa(b)* gene from *B.t.* var. *kurstaki* [2] consisted of a fragment extending from the *Nsi* I site at + 6 (relative to the start of translation) to the *Kpn* I site at + 3654, 187 bp beyond termination of translation. A unique *Bam* HI site four bases upstream of the initial methionine codon was generated by oligonucleotide mutagenesis [1] regenerating the complete open reading frame. The full-length gene was cloned into pIC20H [33] at the *Bam* HI and *Kpn* I sites in the polylinker. The 1.83, 1.6 and 0.57 kb deletion clones were constructed by opening this plasmid at the *Bgl* II site in the polylinker followed by *Bal* 31 nuclease digestion (IBI, New Haven, CT). Blunt ends were generated with deoxynucleotide triphosphates and Klenow fragment and a *Bgl* II linker with stop codons in all three reading frames was inserted (Fig. 1A). The 1.00 kb deletion was constructed by introducing the *Eco* RI fragment extending from nt 367 to 997 into an *Eco* RI subclone containing the first 366 bp of the ICP gene. The 1.83 kb deletion was determined to encode a fully toxic peptide using insect bioassays and DNA sequencing. The sizes of the 1.6 and 0.57 kb clones were estimated by restriction enzyme digestion and agarose gel electrophoresis, and are not toxic in insect bioassays.

Bam HI/*Bgl* II fragments of the pIC20H deletions as well as the full-length gene were cloned into the unique *Bam* HI site of vector pIC35/A. The CaMV 35S promoter in pIC35/A (Fig. 1B) contains nt − 941 to − 459 and − 349 to + 9 of the minimal promoter [40] excised with *Hin*d III and inserted into the *Bam* HI site of pUC13 using a *Bgl* II linker. The promoter fragment was restricted with *Sma* I and *Hin*d III and inserted into pIC19R [33]. The polyadenylation signal in pIC35/A was inserted downstream of the unique *Bam* HI site. It is derived from a *Hin*c III fragment containing the putative poly(A) addition site for ORF 26 of the TR-DNA (bases 22440–22727 [5]). The *Bgl* II fragments containing the promoter/gene/polyadenylation regions were inserted into pH575 (Fig. 1C), a micro Ti plasmid binary vector [21].

A 3.0 kb *Hin*d III fragment containing the *cryIIIA* gene of the coleopteran active *B. thuringiensis* var. *tenebrionis* (*B.t.t.*) was constructed as described [48]. A subclone containing the *Pst* I/*Bgl* II fragment (pPstSC) was made by further deletion. The first two amino acids (Met-Thr) of the insecticidal 65 kDa *cryIIIA* peptide were lost in pPstSC. Introduction of a synthetic linker at the *Pst* I site of pPstSC replaced these amino acids (pBPS-5). This linker also added a *Bam* HI site and a consensus ribosome binding site immediately upstream of the translational initiation site in pBPS-5. Plasmid pBPS-5 encodes a fully toxic peptide (M. Adang *et al.*, manuscript in preparation). pIC35S/Btt was constructed using the 2.3 kb *Bam* HI/*Bgl* II fragment of pBPS-5 cloned into the *Bam* HI site of pIC35/A. The plasmid pIC35Ocs was previously described [36].

Transgenic tobacco plants

Triparental mating was used to mobilize pH575 constructs into *Agrobacterium tumefaciens* LBA4404 harboring helper plasmid pAL4404, which provides *vir* functions. *Nicotiana tabacum* cv. *Xanthi nc* leaf discs were transformed with the engineered *A. tumefaciens*, and kanamycin-resistant plants were regenerated [23].

Tissue culture, protoplast isolation and electroporation

Carrot (*Daucus carota*) cell suspension line WOO-1C (obtained from Dr Tom Hodges, Purdue University) was maintained as previously described [36]. Protoplasts were isolated from suspension cells and electroporated [36] with the following modifications. Carrot protoplasts were co-electroporated with 10 μg p35SOcs marker plasmid and 40 μg of various ICP-pIC35/A constructs per 10^6 cells and were harvested at times indicated post-electroporation. Typically, 4×10^7

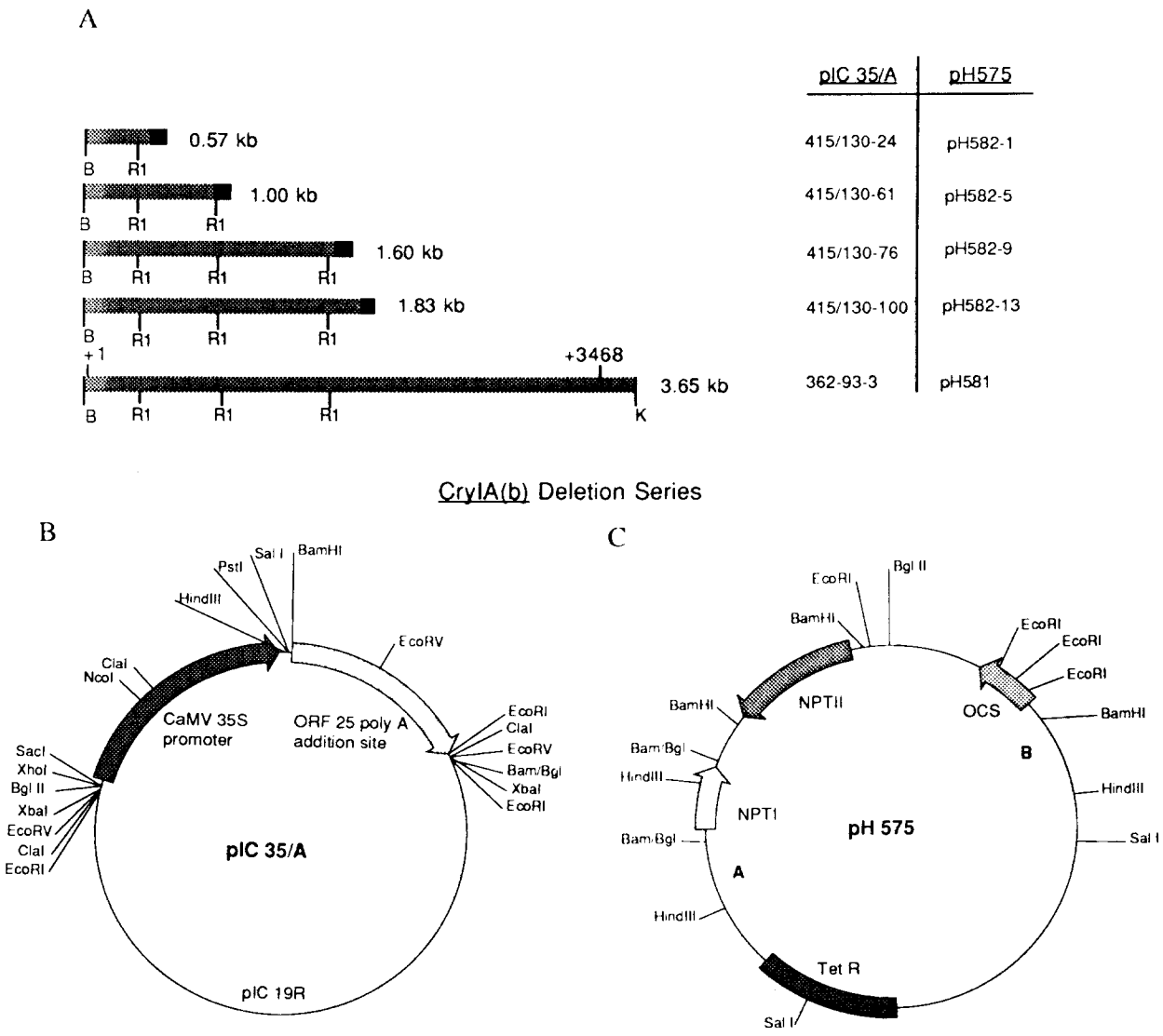

Fig. 1. Construction of *cryIA(b)* deletion series and its insertion into the pIC35/A vector for electroporation and pH575 micro Ti plasmid vector for plant transformation. A. Construction of the deletion series is described in Materials and methods. Stippled areas are the coding region of the *cryIA(b)* gene, solid black areas are the *Bgl* II linker containing stop codons in all three reading frames. + 1 indicates start of transcription and + 3468, termination of transcription in the intact protoxin gene. Agrigenetics Advanced Sciences construct names are given for *cryIA(b)* constructs in both pIC 35/A and pH575. RI, *Eco* RI; B, *Bam* HI; K, *Kpn* I. B. Plasmid map of pIC 35/A. Construction details described in text. *cryIA(b)* deletions were inserted at the *Bam* HI site. C. Plasmid map of pH575. Tet R is the tetracycline resistance gene; NPTI is a bacterial kanamycin resistance gene; NPTII confers kanamycin resistance to plant cells; A and B are 25-nucleotide T-DNA borders; OCS is the octopine synthase gene. ICP genes were inserted at the *Bgl* II site.

cells were electroporated and pooled per time point.

RNA purification and northern blots

RNA was isolated from tobacco leaves pooled from 10 to 30 individual transformed plants per construct. Fresh leaves were homogenized in ice-cold RNA buffer [37] in a Bellco ground glass tissue homogenizer and sequentially phenol- and chloroform-extracted. High-molecular-weight RNA was precipitated with ammonium acetate and poly(A)$^+$ selected as described previously [36]. RNA was isolated from electroporated carrot cells using the same procedure. The RNA samples from carrot cells were DNase-treated to remove residual plasmid DNA, and northern blots were performed [36]. Gel-purified OCS and ICP DNA fragments nick-translated to a specific activity of 5×10^8 cpm with α-^{32}P dATP and dCTP were used as probes. Autoradiography was at -70 °C with intensifying screens for one to three days.

RNA protection assays

DNA probes for RNA protection assays were prepared by restricting plasmids p35SOcs and 362-93-3 (see Fig. 1) with *Eco* RI and end-labeling with T4 polynucleotide kinase (Promega, Madison, WI) and γ-^{32}P-ATP following an alkaline phosphatase treatment. DNA probes were electrophoresed on non-denaturing 5% polyacrylamide gels, appropriate bands excised and the DNA eluted from the crushed gel [55]. The OCS probe (1150 bp band) contained the 35S promoter and the first 275 bp of the OCS gene, and the ICP probe (1135 bp band) had the 35S promoter and the first 269 bp of the ICP gene. RNA protection assays were performed using mung bean nuclease (Epicentre Technologies, Madison, WI) following the method of Murray [38] and electrophoresed on 8% polyacrylamide denaturing gel.

Results

CryIA(c) *gene expression in tobacco*

Initially, we regenerated transgenic tobacco plants containing either the 3.7 kb or a 3′-truncated 2.7 kb *cryIA(c)* gene under the regulation of the mannopine synthase promoter [1]. Northern blots from poly(A)$^+$ RNA purified from young plants only showed hybridization to a truncated, 1.7 kb transcript. Southern blots indicated these genes were intact [1]. When the same poly(A)$^+$ RNA is hybridized with NPTII or OCS probe, these transcripts were present at levels 100-fold greater than the aberrant ICP transcripts. As the plants matured, the 1.7 kb transcript was no longer observed, nor was it seen in their progeny. These plants were not ICP ELISA positive, although individual plants and their progeny were toxic to *M. sexta* in insect bioassays [1]. In an effort to determine whether these genes were turned off due to DNA methylation as the plants matured, DNA was prepared from two transgenic plants shortly after truncated *cry* transcripts were detected and from mature plants which did not express this transcript. The *cryIA(c)* genes were intact in Southern blots and did not differ in their methylation patterns when digested with *Msp* I and *Hpa* II (data not shown). These plants were returned to culture in magenta boxes, but did not resume expression of the truncated transcripts. *CryIA(c)* constructs containing full-length and 3′-truncated *cryIA(c)* genes under the control of other plant promoters (phaseolin, CaMV 35S and 19S, and soybean ribulose 1,5-biphosphate small subunit, with and without its transit peptide) were also introduced into tobacco. Only constructs containing the mannopine synthase promoter were positive for ICP RNA in dot blot assays using 10 μg of total RNA from young plants, so this expression and its subsequent disappearance with plant maturation may be regulated in this promoter.

CryIA(b) *gene expression in tobacco*

To determine whether the low level of ICP RNA expression was unique to the *cryIA(c)* gene, a *cryIA(b)* ICP was introduced to transgenic plants. Isolation and sequence analysis of this gene, HD-1 5.3 gene from *B.t.* var. *kurstaki*, has been described [2]. Initially, two versions of this gene were introduced into pH575 behind the CaMV 35S promoter: the full-length gene, including approximately 200 bp of ICP 3′ untranslated sequence and polylinker region before the ORF25/26 plant polyadenylation sequence; and a truncated gene of 1.83 bk with only ORF 25/26 sequences present in the 3′ end. The latter was toxic to *M. sexta* in insect feeding assays, although a further deletion of only 7 amino acids was not. Due to position effects, the expression levels of transferred genes varies between independent transgenic plants. In order to normalize for these effects, RNA for northern analysis was purified from pools of 15 to 30 independent transformants.

Figure 2 shows a northern blot of poly(A)$^+$ RNA from *cryIA(b)* containing plants. Including poly(A) tract, the intact transcript from the full-length construct is estimated to be 3.7 kb, and from the 1.83 kb deletion, 2.0 kb. Hybridization with ICP probe indicates *cryIA(b)* transcripts from both the 3.65 kb intact gene (lane 2) and the 1.83 kb deletion (lane 1) are degraded. Diffuse bands of approximately 1.6 and 0.9 kb can be detected in lanes 1 and 2. RNA purified from plants transformed with the pH575 construct alone (lane 3) does not hybridize to ICP probe. Lane 5 contains 43 pg of a 1.2 kb *in vitro* synthesized ICP transcript. When this blot was rehybridized with OCS probe, the OCS transcript appeared intact, indicating RNA in the preparation was not degraded (data not shown). Levels of ICP transcript were also significantly lower than OCS in these samples (data not shown). Occasionally, individual transgenic plants were analyzed, and when ICP RNA was detected, they showed a similar pattern of shorter transcripts. We were not successful in mapping the 5′ end of the ICP transcript in poly(A)$^+$ RNA from either pooled or individual plants, possibly due to its low level and degraded nature.

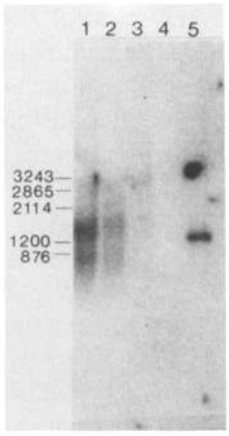

Fig. 2. Expression of *cryIA(b)* gene in tobacco plants. Northern blot of 5 μg poly(A)$^+$ RNA from 15 to 30 pooled transgenic tobacco plants. Lane 1, 1.83 kb deletion (pH582-13); Lane 2, 3.65 kb intact gene (pH581); Lane 3, pH575 with no inserted DNA; Lane 4, blank; Lane 5, 48 pg 1.2 kb ICP RNA transcribed from sp65 vector. BMV size markers are shown on the left.

Expression of 1.83 kb ICP cryIA(b) *construct in electroporated carrot protoplasts*

Using transgenic plants, we were only able to measure steady-state levels of mRNA accumulation. In order to test whether RNA stability was a factor in the low level of ICP expression in plants, we electroporated carrot protoplasts with plasmid constructs containing ICP genes and 35S promoters. Plant protoplasts can be stimulated by electroporation to take up DNA, permitting transient expression of foreign genes. Most genes introduced by electroporation are transcriptionally active for only a short period [44]. Analysis of RNA levels at time points after electroporation permits estimation of the kinetics of gene expression. If the truncated poly(A)$^+$ transcripts observed in transgenic plants are the product of premature termination of transcription or inappropriate polyadenylation, only the same small amounts of these transcripts should be present in RNA purified from electroporated

cells. However, if these transcripts are stable intermediates in the RNA degradation process, RNA from electroporated cells should contain full length products of transcription, with more degradation at later time points in the experiment. Electroporation conditions, optimized for maximum expression of a chimeric CaMV 35S chloramphenicol transferase (CAT) construct using CAT assays were used in the present studies [36]. Initially, 10 μg of plasmid containing the 1.83 ICP deletion construct was co-electroporated with 10 μg of the control plasmid p35SOcs per 10^6 protoplasts. Cells were harvested at 12, 24, 36 and 48 h after electroporation. Poly(A)$^+$ RNA was prepared from 3×10^7 cells per time point. 1 μg was electrophoresed and transferred to northern blots in replicate for hybridization to OCS and ICP probes. Poly(A)$^+$ OCS RNA was readily detected. The highest level was at 12 h, with a small amount present 48 h after electroporation [36]. ICP RNA was only detected in the 12 h time point after long exposure (data not shown).

Earlier experiments showed levels of CAT activity and OCS RNA expression increase linearly with the amount of plasmid introduced, up to 100 μg/10^6 protoplasts (B. Bowen, unpublished results). In subsequent experiments, 40 μg of the various ICP constructs was electroporated per 10^6 protoplasts, and 10 μg of pIC35SOcs control plasmid. Protoplasts were electroporated with the 1.83 ICP deletion cloned into pIC35/A and with p35SOcs.

Figure 3 shows the results of northern blot analyses of poly(A)$^+$ RNA prepared from cells harvested at 1, 2, 4, 8 and 18 hours after electroporation. The level of ICP RNA (lanes 2–7) in these electroporated cells is much lower than control OCS RNA (lanes 8–13), even though 4-fold higher levels of plasmid were electroporated and 10-fold higher levels were electrophoresed. Strikingly, the major ICP transcript at the earlier time points (lanes 3–6) is the size predicted for full-length, polyadenylated RNA in this construct (approximately 2 kb). The distinct shorter fragments observed in transgenic plants are also detected (lanes 3–6) and may represent

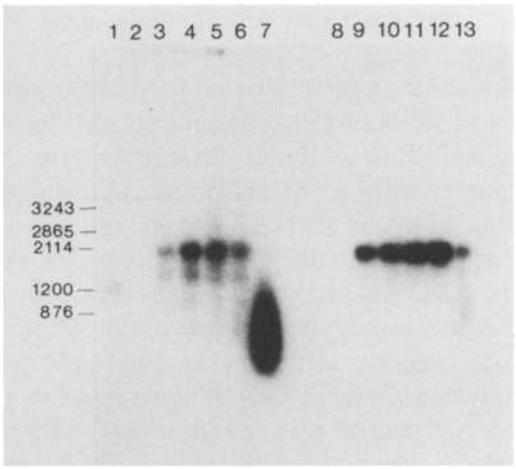

Fig. 3. Time course of poly(A)$^+$ RNA appearance. Northern blot of 1 μg (lanes 2–7) or 0.1 μg (lanes 8–13) poly(A)$^+$ RNA prepared at indicated times from carrot protoplasts co-electroporated with 40 μg of 1.83 kb *cryIA(b)* deletion in pIC 35/A (415/130-100) and 10 μg p35SOcs. Lanes 1–7 were hybridized to nick-translated ICP probe and lanes 8–13 with nick-translated OCS probe as described in Materials and methods. Lane 1, 48 pg 1.2 kb ICP RNA transcribed from sp65 vector; Lanes 2 + 8, carrot RNA; (Lanes 3 + 9, Lanes 4 + 10, Lanes 5 + 11, Lanes 6 + 12, Lanes 7 + 13 had RNA samples prepared 1, 2, 4, 8 and 18 h respectively after electroporation). BMV size markers and 1200 bp size of ICP RNA are shown on the left.

stable intermediates in the RNA degradation process. By 18 h after electroporation (lane 7), a large amount of degraded signal was detected. This resolved into a series of smaller bands in shorter exposures. The OCS signal also began to degrade by 18 h after electroporation (lane 12), although to a lesser extent.

Expression of cryIA(b) *deletions in electroporated carrot protoplasts and transgenic tobacco*

To determine if sequences in the coding region of the *cryIA(b)* contribute to the apparent lack of transcript stability in plants, we constructed a series of 3′ deletions in the pIC35/A vector (Fig. 1). Details of construction are described in Materials and methods. These deletions resulted in 3′ truncation of the ICP gene, with appropriate termination of translation conferred by addition

of a linker containing stop codons in all three reading frames.

We electroporated plasmid prepared from the deletion series as well as the intact 3.4 kb *cryIA(b)* gene into carrot cells as described above, and prepared poly(A)$^+$ RNA from cells harvested at 2 and 18 hours after electroporation. Figure 4 shows a northern blot of RNA prepared from these cells. The predominant ICP transcript detected at 2 hours after electroporation in all deletions was the corresponding full-length transcript (panel A, lanes 3–6), although some shorter transcripts can be seen (panel A, lanes 4–6). No transcript was detected at 2 hours with the intact ICP gene (panel A, lane 7). The amount of ICP message detected at 2 hours was less than OCS message (compare panel A 3–7 with panel B 2–6) since 4-fold higher levels of ICP plasmid were electroporated and 5-fold higher levels were electrophoresed. Deletion of sequences in the 3′ end of the *cryIA(b)* gene did not lead to an increase in message stability in electroporated carrot cells. By 18 hours after electroporation, all ICP hybridization was to degraded RNA, while the OCS transcript was still relatively intact (compare panel A, lanes 8–12 with panel B, lanes 7–11). Variation in OCS levels in panel B are due to pipetting error, since 0.6 μg poly(A)$^+$ RNA was heat-denatured in a single tube for each sample, and 5/6 of the sample loaded for the ICP northern, and the remainder for the OCS northern.

We generated a second 3′ deletion series in pIC35/A using the *cryIA(c)* gene, with the smallest deletion at 1.00 kb. The *cryIA(b)* and *cryIA(c)* genes are highly conserved at their 5′ ends and more divergent at their 3′ ends [2, 22]. The *cryIA(c)* deletions were also electroporated into carrot cells, and no increase in transcript stability was observed in the deletion series.

We also purified poly(A)$^+$ RNA from pools of 15 to 30 tobacco plants transformed with the 0.57, 1.00, 1.6 and 1.87 kb deletions (Fig. 5). In all these constructs, the amount of ICP transcript

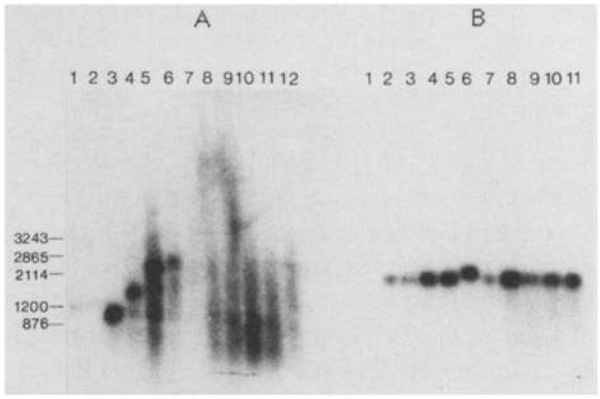

Fig. 4. Time course of RNA appearance in 3′ deletion series. Northern blot of 0.5 μg (panel A) or 0.1 μg (panel B) poly(A)$^+$ RNA prepared from carrot protoplasts co-electroporated with 40 μg of various ICP plasmids and 10 μg of p35SOcs. Cells were harvested at 2 h (A, 2–7; B, 1–6) or 18 h (A, 7–12; B, 7–11) after electroporation. RNA in panel A was hybridized to nick-translated ICP probe and in Panel B, to nick-translated OCS probe. Panel A: lane 1, 48 pg 1.2 kb ICP RNA transcribed from sp65 vector; lane 2, carrot RNA; Lanes 3 + 8, 0.57 kb deletion; lanes 4 + 9, 1.00 kb deletion; lanes 5 + 10, 1.6 kb deletion; lanes 6 + 11, 1.87 kb deletion; lanes 7 + 12, full-length ICP gene. Panel B: lane 1, carrot RNA; lanes 2 + 7, 0.57 kb deletion; lanes 3 + 8, 1.00 kb deletion; lanes 4 + 9, 1.6 kb deletion; lanes 5 + 10, 1.87 kb deletion; lanes 6 + 11, full-length ICP gene. BMV size markers and 1200 bp size of ICP RNA are shown on left.

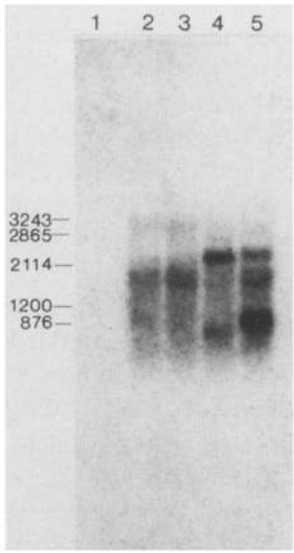

Fig. 5. Expression of *cryIA(b)* deletion series in tobacco. Northern blot of 5 μg poly(A)$^+$ RNA from 10 to 30 transgenic tobacco plants hybridized to ICP probe. Deletion mutation described in text and Fig. 1. Lane 1, blank; lane 2, 1.83 kb deletion; lane 3, 1.60 kb deletion; lane 4, 1.00 kb deletion; lane 5, 0.57 kb deletion. BMV size markers and 1200 bp size of ICP RNA are shown on the left.

was approximately 100-fold lower than for OCS (data not shown). However, the level of ICP transcript in plants containing the 0.57 kb deletion was approximately 5-fold higher than for the other deletions (Fig. 5, compare lanes 2–4 with lane 5). This result suggests that while shortening the *cryIA(b)* gene to 0.57 kb results in a more stable transcript, the level of *cryIA(b)* expression is still approximately 20-fold lower than OCS or NPTII. The smallest deletions (1.00 and 0.57 kb) have larger transcripts as well (Fig. 5, lanes 4, 5). These probably represent read-through transcription from the OCS or NPTII genes [1].

We introduced a third ICP gene into carrot cells with less sequence similarity than the two other ICP genes tested. The *cryIIIA* gene [48] is toxic to coleopteran insects, and encodes a 73 kDa polypeptide. This gene shares only 50.2% sequence homology with the 5′ 1827 nt of the lepidopteran active *cryIA(c)* gene. Figure 6 shows a northern blot of RNA prepared from these cells. Again, the full-length transcript was predominant, and truncated, polyadenylated transcripts were detected. The level of *cryIIIA* transcript relative to OCS control was slightly higher than for *cryIA(b)* or *cryI(c)* constructs.

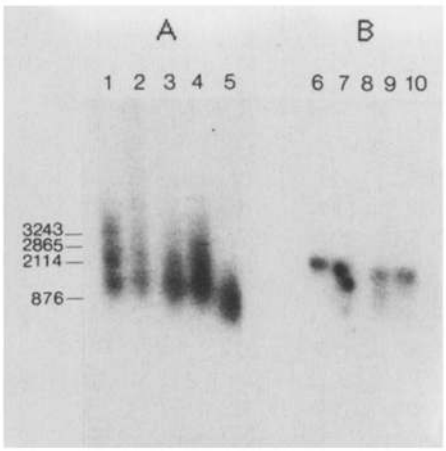

Fig. 6. Time course of expression of *cryIIIA* RNA in electroporated carrot cells. Northern blot of 0.1 μg poly(A)$^+$ RNA prepared from carrot protoplasts co-electroporated with 40 μg of pIC35S/Btt and 10 μg pIC35SOcs, and harvested at times indicated after electroporation. Lanes in panel A were hybridized to nick-translated *cryIIIA* probe and in panel B, to OCS probe. Lanes 1 + 6, 2 + 7, 3 + 8, 4 + 9, 5 + 10 had RNA samples prepared 1, 2, 4, 8 and 18 h after post-electroporation respectively. BMV size markers are shown on the left.

RNA protection of cryIA(b) *transcripts from electroporated cells*

The truncated, polyadenylated *cry* gene transcripts could result from a number of cellular processes. These include efficient polyadenylation at a nonconsensus site within the coding region of the gene, internal initiation of tran-

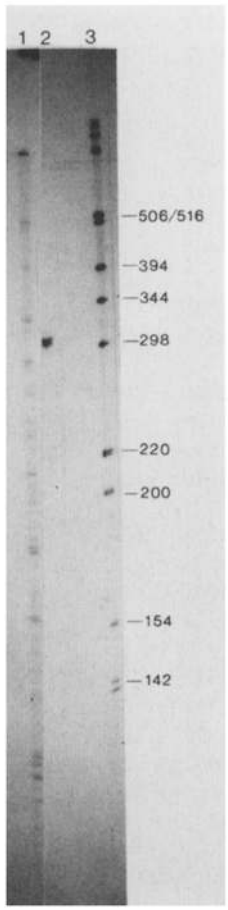

Fig. 7. RNA mapping of the 5′ end of the *cryIA(b)* and OCS transcripts. 1 μg poly(A)$^+$ RNA from 4 h after electroporation sample was hybridized with DNA probe as described in Materials and methods. Lane 1 is *cryIA(b)* probe; lane 2 is OCS probe; lane 3 is kinased kb ladder (BRL, Gaithersburg, MD).

Table 1. Frequency of codon usage in 3 *cry* genes, 207 plant genes (from [40]) and the OCS gene.

a.a.	Codon	Plants		*B.t.*		OCS		a.a.	Codon	Plants		*B.t.*		OCS	
		n	%	*n*	%	*n*	%			*n*	%	*n*	%	*n*	%
Gly	GGG	731	15	48	17	5	20	Trp	TGG	709	100	64	100	4	100
Gly	GGA	1629	32	129	46	6	24	End	TGA	68	33	0	0	1	100
Gly	GGT	1477	29	68	24	6	24	Cys	TGT	432	40	31	63	0	0
Gly	GGC	1179	24	38	13	8	32	Cys	TGC	647	60	18	37	4	100
Glu	GAG	2102	57	76	25	15	60	End	TAG	48	24	2	67	0	0
Glu	GAA	1616	43	234	75	10	40	End	TAA	88	43	1	33	0	0
Asp	GAT	1458	50	167	76	6	50	Tyr	TAT	743	37	161	79	5	56
Asp	GAC	1441	50	54	24	6	50	Tyr	TAC	1267	63	42	21	4	44
Val	GTG	1354	31	54	19	12	41	Leu	TTG	1185	22	32	9	7	19
Val	GTA	491	11	111	39	3	10	Leu	TTA	412	8	154	45	1	3
Val	GTT	1478	34	82	29	8	28	Phe	TTT	1047	40	148	77	8	50
Val	GTC	1045	24	38	13	6	21	Phe	TTC	1597	60	45	23	8	50
Ala	GCG	546	11	46	20	10	25	Ser	TCG	343	8	29	9	4	14
Ala	GCA	1156	22	75	22	14	36	Ser	TCA	768	17	72	22	5	17
Ala	GCT	1901	37	79	25	10	25	Ser	TCT	1009	22	67	21	6	21
Ala	GCC	1548	30	27	12	5	13	Ser	TCC	896	20	46	14	5	17
Arg	AGG	742	26	28	11	6	35	Arg	CGG	198	7	8	3	3	18
Arg	AGA	707	24	113	46	3	18	Arg	CGA	214	7	33	13	0	0
Ser	AGT	581	13	82	25	2	07	Arg	CGT	534	18	52	21	2	12
Ser	AGC	887	20	28	9	7	24	Arg	CGC	520	18	12	5	3	18
Lys	AAG	2241	66	32	24	4	40	Gln	CAG	1465	43	27	16	6	57
Lys	AAA	1139	34	104	76	6	60	Gln	CAA	1912	57	142	84	0	43
Asn	AAT	1137	41	208	73	8	40	His	CAT	575	48	70	89	2	40
Asn	AAC	1646	59	78	27	12	60	His	CAC	625	52	9	11	3	60
Met	ATG	1356	100	40	100	5	100	Leu	CTG	792	15	23	7	4	11
Ile	ATA	505	16	63	25	5	23	Leu	CTA	434	8	59	17	4	11
Ile	ATT	1241	40	133	53	8	36	Leu	CTT	1273	24	62	18	11	31
Ile	ATC	1374	44	54	22	9	41	Leu	CTC	1189	22	12	4	9	25
Thr	ACG	343	11	55	21	7	35	Pro	CCG	492	13	38	20	4	23
Thr	ACA	745	24	104	39	2	10	Pro	CCA	1507	39	90	47	7	41
Thr	ACT	990	31	67	25	6	30	Pro	CCT	1063	28	55	29	3	18
Thr	ACC	1082	34	38	14	5	25	Pro	CCC	755	20	7	4	3	18

scription within the gene, endonucleolytic cleavage at a site within the coding region, exonucleolytic degradation from the 5′ end of the gene, or splicing using cryptic splice sites within the gene. The 5′ ends of the 1.83 *cryIA(b)* deletion construct and the co-electroporated pIC35SOcs was determined using a mung bean nuclease protection assay (Fig. 7) and poly(A)$^+$ RNA from cells 4 h after electroporation. Two protected bands of approximately 305 bp in length were observed using the OCS probe, indicating the normal transcription initiation site of the 35S promoter was used [37]. The predicted size of the ICP transcript using the normal 35S transcription

initiation site is 310 bp. Mapping with the *cryIA(b)* probe revealed many 5′ ends, although a faint band the predicted size is detected. This result is compatible with degradation from the 5′ end of the transcript. Numerous attempts to map the 3′ ends of the short *cryIA(b)* transcripts using internal *Eco* RI DNA fragments and poly(A)$^+$ RNA from electroporated cells and transgenic plants were unsuccessful. If the short polyadenylated *cry* transcripts resulted from use of an internal site within the gene, such probes should have demonstrated this. Our inability to map such ends could be due to the degraded nature of the RNA.

Discussion

We have demonstrated that the metabolism of *cryIA(b)* mRNA differs from that of OCS in transgenic plants and electroporated carrot cells. Although it is well established that individual mRNAs can vary in stability by more than an order of magnitude [45, 46, 49], the molecular basis for regulation of mRNA degradation is poorly understood. However, the mechanisms and rates of mRNA degradation are ultimately directed by the primary structure of the transcript itself.

The stability of some mRNAs may be influenced by their translational efficiency, by the structure or intracellular compartmentalization of the polysomes with which they are associated, or by the level of protein they produce [12]. The abnormal mRNA metabolism we have demonstrated in *cry* genes in plants may be due to the unfavorable translational state of the message. Recently, a number of researchers working on plant genes [25, 47, 59, 60] have observed that mRNAs which prematurely terminate translation are rapidly turned over. Other eukaryotic genes with nonsense or frameshift mutations leading to premature termination of translation also show an increased rate of degradation [7, 8, 12, 20, 30, 31, 32]. Although the *cry* genes do not contain premature stop signals, their codon usage is biased for expression in bacterial cells and may not be optimal for translation in plant cells. Table 1 compares the codon usage of three *cry* genes (*cryIA(b)*, *cryIA(c)* and *cryIII*) with that of 207 plant genes [35] and OCS [5]. The commonly used codons in these *cry* genes are clearly different from those in typical plant genes. In most cases, OCS codon usage is more similar to plants than to *cry* genes. In general, plant codon usage more closely resembles human and other higher eukaryotes than unicellular organisms due to the overall preference for G + C content in codon position III [35]. Bias in codon choice between genes in a single species appears related to the level of protein expression of a given gene. Systematic codon preferences have been reported for highly expressed bacterial and yeast genes [9, 13, 50, 51]. In one group of highly expressed genes in yeast, over 96% of the amino acids are encoded by only 25 of the 61 available codons [9].

Hoekema *et al.* [20] substituted increasing numbers of synonymous minor codons for biased major codons in the yeast gene phosphoglycerate kinase and observed up to a 10-fold decrease in steady state levels of protein and a three-fold decrease in steady-state mRNA levels. They concluded that the rate of mRNA translation influences mRNA stability in yeast. Pedersen [42] determined that the translation elongation rate in *Escherichia coli* of poorly expressed mRNAs such as *lacI* and *bla* is only 50% of that for highly expressed mRNAs like ribosomal proteins. If minor codons are decoded by less abundant tRNA species, ribosomal pausing may occur, destabilizing mRNA in a manner similar to premature termination of translation from a stop codon mutation [20]. However, the mechanism for the decrease in mRNA stability associated with premature stop codons may be more complex than that of a 'poorly translated' gene (M. Culberson, personal communication).

Recently Lim *et al.* [30] have reported on a novel metabolism of several β°-thalassemic β-globin genes. The majority of these mutations are frameshift or nonsense mutations resulting in premature β-globin mRNA translation and unstable mRNAs. Lim *et al.* analyzed the RNA metabolism of transgenic mice containing three different β°-thalassemia mutants and found that each pro-

duced properly processed, abnormally short-lived mRNAs as well as three smaller RNAs. These smaller mRNAs were polyadenylated and lacked the normal mRNA 5′ end. They were only seen in cytoplasmic RNA, indicating they derived from full-length mRNA by a cytoplasmic process dependent on the translational state of the mRNA such as endonucleolytic or 5′ → 3′ exonucleolytic activity. Vancanneyt *et al.* [59] transformed tobacco with patatin genes which prematurely terminate translation. Truncated mRNAs appear on their northern blots of poly(A)$^+$ RNA from transgenic plants which could result from 5′ → 3′ degradation of poorly translated mRNAs.

When we mapped the 5′ end of the *cryIA(b)* transcript in electroporated cells, we observed heterogeneous 5′ ends (Fig. 7). Preliminary results indicate that the ORF 25/26 polyadenylation site is used correctly, and efforts to map internal polyadenylation sites were unsuccessful (data not shown). These results are consistent with the origin of short, polyadenylated transcripts through endonucleolytic or 5′ → 3′ exonucleolytic activity. The diffuse 1.6 kb and 0.9 kb bands observed in northern blots (Figs. 2, 3) may result from pausing of degradation at regions of strong secondary structure within the *cry* coding sequence. Ross has described stable pause sites in the 3′ → 5′ degradation of β-globin transcript *in vitro* [46] and Lim *et al.* [30] suggest similar pause sites are present in its 5′ → 3′ degradation. If this is the case, we predict that improving the translational efficiency of *cry* mRNAs will decrease the level of truncated, polyadenylated mRNA and increase the level of stable, full-length RNA.

The presence of this novel 5′ → 3′ mRNA metabolism does not exclude a role for the well characterized 3′ → 5′ degradation process in the turnover of *cry* mRNAs. The northern blots in which anomalous small RNAs were detected contained poly(A)$^+$ RNA, and transcripts degraded 3′ → 5′ would be excluded after loss of their poly(A) tract. A broad smear of degraded transcripts was detected in northern blots of total RNA from *cryIA(c)* transgenic tobacco (data not shown).

The 5′ untranslated leader of tobacco mosaic virus and alfalfa mosaic virus (AMV) coat protein mRNAs have been shown to enhance the expression of foreign gene transcripts both *in vitro* and *in vivo* [15, 24, 54]. In order to improve translation of the *cryIA(b)* mRNA in plants, we placed the AMV coat protein leader sequence between the 35S promoter and the gene. We also altered the codon context of the initial AUG from CTTATGG to ACCATGG, the eukaryotic consensus sequence derived from a compilation of 211 animal and plant mRNA sequences [28]. Constructs containing C at the −3 position decreased translation two to eight-fold relative to the optimal consensus sequence in transgenic plants [4, 57]. Kozak [29] also showed that in mammalian cells, replacement of a purine by a pyrimidine at the −3 position relative to the first AUG significantly decreased translation, and mutations at −1, −2 and +4 had more pronounced effects, with inappropriate codon context decreasing translation 20-fold. Thus, the context of the initial AUG in our *cryIA(b)* construct was unfavorable to initiation of translation *in vivo*, and our changes should increase its efficiency.

We have shown that addition of viral leader sequences and improvement of the consensus sequence surrounding the first AUG increased the amount of intact mRNA detected in transgenic tomato plants as well as the level of *cryIA(b)* protein [3]. However, these plants continued to produce the truncated, polyadenylated products observed in electroporated carrot cells. Barton *et al.* [6] independently introduced identical modifications to the AUG consensus sequence and the AMV viral leader sequence to their *cryIA(a)* construct, with similar results. These findings suggest that sequences contained within the coding regions of *cry* genes contribute to RNA instability.

Recent experiments have shown that rapidly turned over mRNAs of proto-oncogenes contain sequences which affect their stability [16, 19, 26, 41, 52, 53]. These 'instability elements' may act by dissociating poly(A) binding protein from the poly(A) tract, leaving 3′ ends subject to rapid degradation by exonucleases [10, 11]. Such se-

quences could also direct transcripts to specific cellular compartments, thereby limiting or increasing their exposure to *trans*-acting factors such as sequence-specific endonucleases. The *cry* genes introduced to plants by our group and others are extremely AT-rich compared with the majority of plant genes. In *B.t.*, the *cry* mRNAs are exceptionally stable, in part due to 'retroregulatory sequences' which mediate interactions between the 3′ untranslated region and the 5′ end of the message [62]. But in plants, sequences in the coding region of the *cry* genes may act as instability elements leading to rapid degradation of these mRNAs. Although the mechanisms by which such instability elements within genes promote mRNA decay are unknown, there appears to be a close link with the translation process.

We have placed the *cryIA(b)* gene under the control of the 2019E soybean heat shock (HS) promoter [4] (A. Merlo and M. Adang, manuscript in preparation). Under heat shock conditions, transcription from genes lacking the HS promoter ceases, and only HS mRNA is translated [27]. However, non-HS mRNAs are stable during heat shock. It is likely that the enzymes responsible for normal mRNA turnover in cells are short-lived and require ongoing protein synthesis to maintain effective levels. Several groups have observed that inhibition of protein synthesis by cycloheximide, anisomycin, heat shock or sodium arsenite leads to increased stability of the rapidly turned over mRNAs of proto-oncogenes such as c-*fos* and c-*myc* [11, 17, 18, 34, 56]. High levels of *cryIA(b)* full-length transcript was detected after heat shock in both transgenic tobacco plants and electroporated carrot cells, and the short, polyadenylated transcripts were missing. The *cryIA(b)* mRNA was transiently transcribed, as predicted for a foreign gene under the control of a HS promoter, and was not stable after the heat shock wore off. It is possible that *cry* genes are unstable in plants because their coding regions contain RNA instability sequences. When *cry* mRNAs are inefficiently translated, these RNA instability elements are not protected by translating ribosomes, and the mRNA is quickly degraded.

Although these instability elements may be contained throughout the *cry* genes, some are predicted to be within the first 570 bases of the *cryIA(b)* gene. In carrot cells, the 0.57 kb deletion was not expressed at higher levels 2 hours post-electroporation (Fig. 4 panel A, lane 3) and was still degraded at 18 h after electroporation relative to the OCS transcript (compare Fig. 4 panel A lane 8 with panel B lane 7). The 0.57 kb deletion did produce approximately five-fold higher levels of *cry* RNA in transgenic tobacco plants (Fig. 5). However, signals of this intensity could be detected with 20-fold less poly(A)$^+$ RNA and either OCS or NPTII probes (data not shown). Experiments are currently underway to rebuild various *cry* genes for maximum translation in plants, replacing rare codons with synonymous codons more commonly used in highly expressed plant genes. In the course of rebuilding these *cry* genes to correct for codon bias, these putative instability sequences may be identified and removed.

After this manuscript was submitted, Perlak *et al.* [42] published a paper describing insect-resistant cotton plants containing *cryIA(b)* or *cryIA(c)* truncated structural genes in which the coding sequence was modified without altering the encoded amino acid sequence. The changes were designed to alter potential regulatory sequences, predicted mRNA secondary structure and codon usage. These sequences were significantly altered, retaining less than 80% homology to the wildtype DNA sequences. The altered genes expressed 50- to 100-fold more protein than the original genes in the same expression vectors and greater levels of insect toxicity. The effect of these modification on *cry* mRNA stability is not discussed, although our results predict the modified *cry* mRNAs are significantly more stable than the original messages. Whether this rebuilt gene functions better due to improved codon bias and more efficient translation or whether instability sequences have been removed remains to be determined.

Acknowledgements

We thank John McCron for preparation of carrot protoplasts, Chris Scott, Joe Lutz, and Ann Merlo for help in electroporation, Dave DeBoer for tobacco transformation, Kay Rashka for ELISAs, Janet Klein for insect bioassays, Don Merlo, Glen Staffeld, Wally Buchholz and Tom Franz for vector construction of pIC35S/a and pH575, Jane Pertzborn for maintaining greenhouse plants, and Chuck Armstrong, Les Hoffman, Deb Donaldson and Ben Bowen for helpful discussions.

This research was supported by Agrigenetics Advanced Sciences Corporation, Madison WI and Public Health Service grant CA-22443. E.E.M. was supported by Public Health Service Training Grant CA-9075.

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