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A synthetic *cryIC* gene, encoding a *Bacillus thuringiensis* δ-endotoxin, confers *Spodoptera* resistance in alfalfa and tobacco

(armyworms/*CryIC* δ-endotoxin/gene synthesis/transgenic alfalfa)

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ABSTRACT ***Spodoptera* species, representing widespread polyphagous insect pests, are resistant to *Bacillus thuringiensis* δ-endotoxins used thus far as insecticides in transgenic plants. Here we describe the chemical synthesis of a *cryIC* gene by a novel template directed ligation–PCR method. This simple and economical method to construct large synthetic genes can be used when routine resynthesis of genes is required. Chemically phosphorylated adjacent oligonucleotides of the gene to be synthesized are assembled and ligated on a single-stranded, partially homologous template derived from a wild-type gene (*cryIC* in our case) by a thermostable *Pfu* DNA ligase using repeated cycles of melting, annealing, and ligation. The resulting synthetic DNA strands are selectively amplified by PCR with short specific flanking primers that are complementary only to the new synthetic DNA. Optimized expression of the synthetic *cryIC* gene in alfalfa and tobacco results in the production of 0.01–0.2% of total soluble proteins as CryIC toxin and provides protection against the Egyptian cotton leafworm (*Spodoptera littoralis*) and the beet armyworm (*Spodoptera exigua*). To facilitate selection and breeding of *Spodoptera*-resistant plants, the *cryIC* gene was linked to a *pat* gene, conferring resistance to the herbicide BASTA.**

Insecticidal Cry proteins, produced as protoxins (65–140 kDa) in parasporal crystals of *Bacillus thuringiensis* (*Bt*), are active as selective entomocidal agents. The crystalline *Bt* protoxins are solubilized and activated in the midgut of insects by proteolysis. The activated toxins (60–70 kDa) bind to the membrane of midgut columnar cells and form ion channels, inducing osmotic lysis of the epithelium (1–3). Engineering of insect resistance in maize, rice, cotton, tomato, potato, and tobacco shows that a significant modification of the bacterial *cry* coding sequences is essential to express these *Bt* toxin genes in plants (4–11). Efficient transcription of recombinant *cry* genes in plant cell nuclei was achieved by the removal of A+T-rich sequences that may cause mRNA instability (8–11) or aberrant splicing (12), and the translation of *cry* mRNAs is enhanced by modification of their codon usage to make it more similar to that of the host plant (8). Expression of bacterial *cry* genes in chloroplasts may overcome these technological demands; however, to date chloroplast transformation is only available in tobacco (13).

The insecticidal spectrum of *Bt* toxins thus far expressed in transgenic plants is limited. Therefore, the engineering of *Bt* toxins with novel specificity is essential for the biological control of recalcitrant plague insects, such as *Spodoptera*. Members of the *Spodoptera* genus (Lepidoptera, Noctuidae) feed on over 40 different plant families world-wide, including at least 87 species of economic importance (14). Studies of *Spodoptera*-specific *Bt* isolates indicated that many of them synthesize CryIC δ-endotoxins that are much more effective against these insects than other CryI proteins (15). Here we describe the chemical synthesis of a gene coding for a truncated CryIC protoxin of 630 amino acids and demonstrate that its expression in alfalfa and tobacco provides resistance to the Egyptian cotton leafworm (*Spodoptera littoralis*) and the beet armyworm (*Spodoptera exigua*).

MATERIALS AND METHODS

Construction of a Synthetic *cryIC* Gene. Oligonucleotides were synthesized with an Applied Biosystems 380B DNA synthesizer, using a 1000 Å controlled pore glass support (Millipore). Except for primers located at the 5′ termini of template directed ligation (TDL)-PCR sequence blocks (see below), the 5′ ends of oligonucleotides were chemically phosphorylated with 5′-Phosphate-ON phosphoramidite (CLONTECH) in the final step of DNA synthesis. The synthesis products were deprotected with ammonium hydroxide, desalted on NAP25 (Pharmacia) columns, and used for TDL-PCR without additional purification. Design of the synthetic *cryIC* gene was based on the sequence of the corresponding wild-type gene (*cry1Ca5*; GenBank accession no. X96682) (16). In fact, the *cry1Ca5* sequence represents a consensus of all known *cryIC* genes and is identical in three *Bt* strains, K26-21, MR1-37, and ssp. *aizawai* 7.29 (16). Modifications of the synthetic *cryIC* gene (*s-cryIC*) sequence did not alter the amino acid sequence of the minimal toxic fragment of the CryIC protoxin. The designed DNA sequence of the *s-cryIC* gene (see Fig. 1; GenBank accession no. X99103) was divided into three blocks separated by *Hin*cII and *Bgl*II cleavage sites. The *Bam*HI–*Hin*cII block I was constructed from eight, the *Hin*cII–*Bgl*II block II from five, and the *Bgl*II–*Bam*HI block III from seven oligonucleotides. The oligonucleotides were assembled on a single-stranded DNA template of phagemid pR1, carrying the 630 N-terminal codons of the wild-type *Bt cryIC* gene (see Figs. 1 and 2). Terminal oligonucleotides in each TDL-PCR block carried unique sequences on their 5′ and 3′ ends, which were not complementary with the template but were matched to short PCR primers for selective amplification of the synthetic DNA strand. These PCR primers contained unique restriction enzyme cleavage sites used for cloning of the amplified double-stranded DNA fragments into pBluescript. The TDL-PCR block I was PCR-amplified by a 5′ primer (5′-AAGAGGATCCACCATGGAGGAGAAC-3′) carrying

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Abbreviations: *Bt*, *Bacillus thuringiensis*; TDL, template directed ligation; CaMV, cauliflower mosaic virus.

Data deposition: The sequence reported in this paper has been deposited in the GenBank data base (accession no. X99103).

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a *Bam*HI site, and a 3′ primer (5′-ATGATCTAGATGCAG-TAGCG-3′). The 3′ primer was complementary to an oligonucleotide (5′-GTCAACTAACAAGGGAAGTTTATACG-GACCCACGCTACTGCATCTAGATCAT-3′) at the 3′ end of block I that carried *cryIC* sequences with the *Hin*cII site and unrelated overhang sequences with an *Xba*I site. The oligonucleotide at the 5′ end of block II (5′-GATAACTCGAGC-GAGCCTAAACTATGACAATAGGAGATATCCAATTC-AGCCAGTTG-3′) added unique DNA sequences with an *Xho*I site to the *cryIC* sequences upstream of the *Hin*cII site and matched a PCR primer (5′-GATAACTCGAGCGAGCC-TA-3′). The 3′-terminal oligonucleotide in block II carried *cryIC* sequences extending to the *Bgl*II site and downstream overhang sequences with an *Xba*I site that were complementary to a PCR primer (5′-CCTGACTCTAGAAGATC-3′). In the oligonucleotide located at the 5′ end of block III, an *Eco*RI site was added upstream of the *Bgl*II site of *cryIC* gene, fitting to a PCR primer (5′-CTGTCTGAATTCAAAGATC-3′). The oligonucleotide at the 3′ end of block III carried a *Bam*HI site, following the position of TAG stop codon in the pR1 phagemid, as well as adjacent unique sequences with a *Not*I site that were complementary to a PCR primer (5′-AGCATGCGGCC-GCGGATCC-3′).

TDL reactions were carried out at a template/oligonucleotide ratio of 1:200 (a total of 0.05 pM of template versus 10 pM of each oligonucleotide) in a final volume of 50 μl, using a reaction buffer (20 mM Tris·HCl, pH 7.5/20 mM KCl/10 mM $MgCl_2$/0.1% Nonidet P-40/0.5 mM rATP/1 mM DTT) and 4 units of *Pfu* DNA ligase (Stratagene). Thirty cycles of TDL were performed at 92°C for 1 min followed by 52°C for 3 min. To increase the number of TDL cycles to 60, a new portion of rATP (0.5 mM) and 4 units of *Pfu* ligase was added. Five microliters from the TDL reaction mix served as template for PCR amplification with 100 pM of primers, 250 μM dNTP, and 2.5 units of Ampli-*Taq* DNA polymerase (Perkin–Elmer) in 100 μl of buffer (10 mM Tris·HCl, pH 9.0/50 mM KCl/0.1% Triton X-100), using of 30 cycles at 92°C for 1 min, 45°C for 1 min, and 72°C for 1.5 min. The amplified DNA fragments were gel-purified, digested with *Bam*HI–*Xba*I (block I), *Xho*I–*Xba*I (block II), and *Eco*RI–*Not*I (block III), then cloned in pBluescript SK(+) to verify their DNA sequences. The errors (three small deletions, one transversion, and one transition) found in the TDL-PCR products were corrected by site-directed mutagenesis using a USE kit (Pharmacia) or by assembly of nonmutated restriction fragments.

Plant Gene Expression Constructs and Transformation of Alfalfa and Tobacco. The plant expression vector pPCV91 was constructed by modification of pPCV720 (17). A *Not*I site in the RK2-domain was eliminated by filling in with DNA polymerase Klenow fragment, and a cauliflower mosaic virus (CaMV) 35S promoter (18) with four repeats of the enhancer domain (−90 to −418) was introduced into the *Hin*dIII site of pPCV720. Upstream of a *Bam*HI cloning site, this cassette contained 20 bp from the 3′ end of the untranslated Ω leader sequence of tobacco mosaic virus RNA (19), whereas downstream of the *Bam*HI site, it carried a polyadenylylation signal sequence derived from the CaMV 35S RNA gene. A *Bam*HI site present in the mannopine synthase promoter (pmas) of pPCV720 was replaced by a *Not*I site using a *Sau*3A–*Not*I adaptor (5′-GATCTGCGGCCGCA-3′). The resulting vector, pPCV91, carried three plant gene expression cassettes with unique *Bam*HI, *Not*I, and *Sal*I cloning sites. To construct pNS6, the synthetic *cryIC* gene was cloned as a *Bam*HI fragment downstream of the CaMV 35S promoter (see Fig. 3*A*). In pNS7, a synthetic *pat* gene, encoding a phosphinothricine acetyltransferase (20), and a *chiAII* gene from *Serratia marcescens* (21) were inserted into the *Sal*I and *Not*I sites located downstream of the *mas* 1′ and 2′ promoters, respectively. A bacterial *cryIC* gene from *Bt* ssp. *aizawai* 7.29 (GenBank accession no. X96682), carrying the 756 N-terminal codons of *cryIC*, was cloned in pGIF1, in which it replaced the synthetic *cryIC* gene of pNS7. Vectors pNS6, pNS7, and pGIF1 were conjugated to *Agrobacterium tumefaciens* GV3101-(pMP90RK) (17) and used for transformation of alfalfa (*Medicago sativa* L. var. Regen S clone RA3) and tobacco (*Nicotiana tabacum* SR1) as described (17, 22). To select for transformed explants, alfalfa and tobacco tissue culture media contained, respectively, 40 μg/ml and 15 μg/ml hygromycin.

Monitoring the Expression of CryIC in Transgenic Plants. Bacterial and synthetic *cryIC* genes, coding for the 630 N-terminal amino acids of the CryIC toxin (see Fig. 1), were cloned into the *Bam*HI site of a pAEN4 vector carrying the CaMV 35S gene expression cassette of pPCV91. *Arabidopsis thaliana* protoplasts were isolated from root cultures and transformed by polyethylene glycol-mediated DNA uptake (23), using 1.5×10^6 protoplasts and 35 μg of plasmid DNA in each experiment. The protoplasts were harvested 48 hr after DNA uptake and lysed in SDS sample buffer to separate proteins on SDS/10% polyacrylamide gels before immunoblotting. An antibody used for immunoblotting was raised against a truncated CryIC δ-endotoxin carrying 756 N-terminal amino acids. Expression of CryIC in *Escherichia coli* strains, carrying bacterial or synthetic *cryIC* genes, respectively, in pET-11a or pET-11d (24), was monitored by a second alkaline phosphatase-conjugated goat anti-rabbit antibody. Immunoblot analysis of proteins synthesized in plant cells was performed using an enhanced chemiluminescence kit (Amersham).

RNA (20 μg) samples isolated from leaves and petioles of alfalfa plants were separated on agarose-formaldehyde gels (25). *Bam*HI fragments (1.9 kb), carrying either synthetic or bacterial *cryIC* sequences (see Fig. 1), and a *Not*I fragment with the *chiAII* gene (1.8 kb) were labeled by random priming and used as hybridization probes.

Insect Bioassays. Leaf bioassays were performed with the Egyptian cotton leafworm (*S. littoralis*) and the beet armyworm (*S. exigua*) using neonate, second to third, third to fourth, and fourth to sixth instar larvae. Ten larvae of a selected developmental stage were placed on a moistened filter disc in Petri dishes with detached leaves from greenhouse grown plants. The assays were repeated two to three times for each plant. The mortality of neonate larvae was scored after 3 days, whereas the mortality of larvae from second to fourth and from fourth to sixth instar stages were evaluated, respectively, after 5 and 7 days. For the insect assays with whole plants, transgenic greenhouse-grown alfalfa lines producing 0.02–0.1% of total soluble protein as CryIC and *S. exigua* larvae of the third to fourth instar stage were used. Three NS7 and three NS6 transgenic, as well as wild-type, plants were infested with 15–20 larvae each. In "free-choice" experiments, 25 larvae were placed in a Petri dish located between transgenic NS6 or NS7 and nontransgenic alfalfa plants in the greenhouse. Leaf damage was evaluated after 6 days.

RESULTS

Synthesis of a *cryIC* Gene Using TDL. A synthetic *cryIC* gene coding for an N-terminal protoxin fragment of 630 amino acids was designed (Fig. 1) by exchanging 286 bp of the bacterial *cryIC* sequence (GenBank accession no. X96682; 1890 bp) such that 249 out of 630 codons were modified according to preferential codon usage in dicotyledonous plants. These exchanges removed 21 potential plant polyadenylylation signals (27), 12 ATTTA motifs, 68 sequence blocks with 6 or more consecutive A/Ts, and all motifs containing five or more G+C or A+T nucleotides. Sequences around the translation initiation site were changed to conform to the eukaryotic consensus sequence (26), and a TAG stop codon was introduced downstream of amino acid codon 630. The G+C

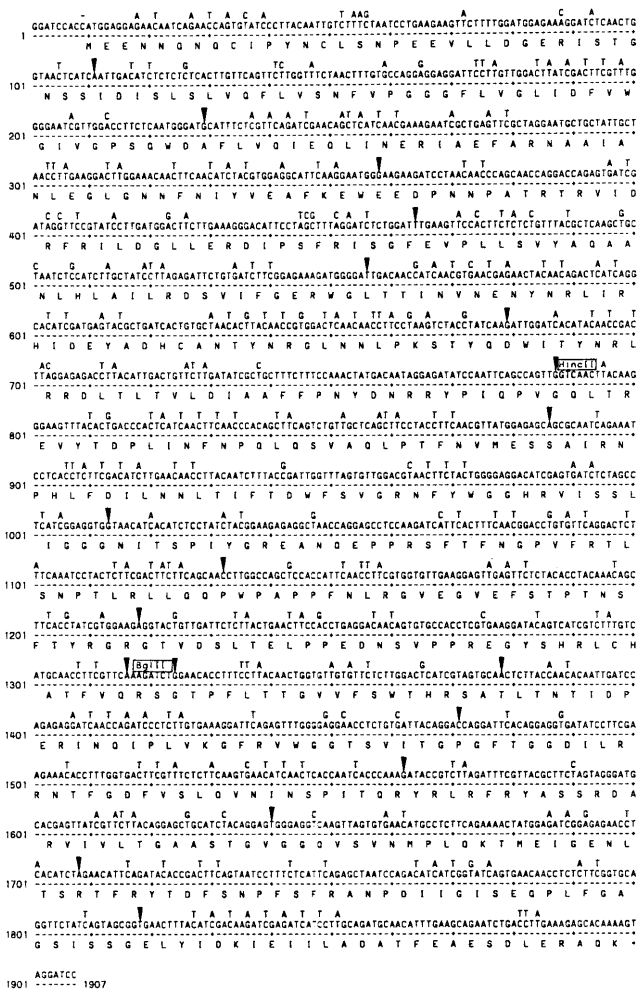
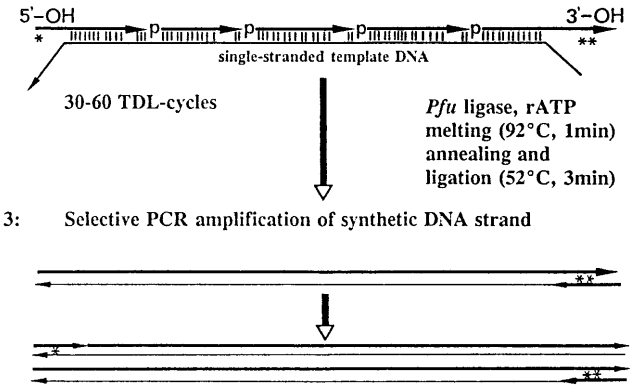


FIG. 1. Nucleotide sequence of the synthetic *cryIC* gene (*s-cryIC*). Nucleotides of the bacterial *cryIC* sequence (*b-cryIC*) exchanged in the synthetic gene are shown in the upper lanes. The nucleotide sequence of the *s-cryIC* region coding for 630 codons starts with an ATG codon in a sequence context fitting the eukaryotic consensus (26) and terminates at a TAG stop codon. Arrowheads above the *s-cryIC* sequence indicate the boundaries of adjacent synthetic oligonucleotides used for TDL-PCR gene synthesis. *Hin*cII and *Bgl*II cleavage sites used for the assembly of three TDL-PCR blocks are indicated by boxes above the sequences.

content of the *cryIC* gene was thus increased from 36.6% to 44.8%. The *s-cryIC* gene was synthesized from oligonucleotides of 70–130 bases that were chemically phosphorylated at their 5′ ends. Because chemical phosphorylation is performed as the last step of automated DNA synthesis, only full-length oligonucleotides contain the 5′ phosphate group. Bacterial *cryIC* sequences coding for the 630 N-terminal codons were cloned in a pBluescript vector to generate a single-stranded DNA template for ordered annealing of five to eight synthetic oligonucleotides by partial base pairing. The adjacent oligonucleotides were assembled and ligated on this single-stranded template by a thermostable *Pfu* ligase using 30–60 cycles of repeated melting, annealing, and ligation. In combination with chemical phosphorylation, this TDL method (Fig. 2) provided a sequence-specific selection for phosphorylated full-length oligonucleotides from a complex mixture of nonphosphorylated failure synthesis products, and yielded a linear amplification of single-stranded synthetic *cryIC* DNA segments generated by ligation. Therefore, except for desalting, no additional purifications of a crude oligonucleotide mixture after chemical DNA synthesis were necessary. The TDL at high temperatures also circumvented potential problems of erroneous annealing. The synthetic *cryIC* sequences were converted to double-stranded DNA fragments and specifically amplified by PCR using short end-primers that did not anneal to the bacterial *cryIC* template carried by the pBluescript vector. The *s-cryIC* gene was thus synthesized from three sequence blocks that were combined by ligation of *Hin*cII- and *Bgl*II-digested DNA fragments and cloned in pBluescript (see *Materials and Methods*).

1: Automated synthesis of 5′-phosphorylated oligonucleotides

2: Template directed ligation of oligonucleotides annealed with a partially complementary single-stranded DNA carrying bacterial *cryIC* sequences



3: Selective PCR amplification of synthetic DNA strand

FIG. 2. Gene synthesis by TDL–PCR. Chemically phosphorylated synthetic oligonucleotides, corresponding to adjacent segments of the *s-cryIC* gene, are annealed with a single-stranded template DNA, carrying partially complementary sequences of the bacterial *b-cryIC* gene. The oligonucleotides are ligated on the template by a thermostable *Pfu* ligase using repeated TDL cycles of melting, annealing, and ligation. The synthetic DNA strand is selectively amplified by short PCR end primers that represent unique terminal sequences of *s-cryIC* oligonucleotides located at the 5′ and 3′ termini of TDL-PCR sequence blocks (labeled by asterisks). These PCR end primers contain suitable restriction cleavage sites for cloning of the synthetic double-stranded DNA fragments.

Expression of the Synthetic *cryIC* Gene in *E. coli*, *Arabidopsis*, Tobacco, and Alfalfa. Bacterial and synthetic *cryIC* genes were cloned, respectively, in vectors pET-11a and pET-11d (24) and expressed in *E. coli*. Synthesis of the CryIC protein was monitored by immunoblotting. In comparison with cells harboring the bacterial *cryIC* gene, the expression of the synthetic gene in *E. coli* yielded a significantly lower toxin level (Fig. 3*B*).

The native and synthetic *cryIC* genes were inserted between promoter and polyadenylylation signal sequences of the CaMV 35S RNA gene in the plant gene vector pAEN4. In pAEN4, the 5′ ends of *cryIC* genes were fused to the untranslated Ω leader sequence of tobacco mosaic virus (19) to enhance the translation of mRNAs, whereas the upstream CaMV 35S promoter was supplemented with four repeats of the enhancer domain (−90 to −418) to stimulate the transcription of chimeric genes in plants (18). The *cryIC* genes were introduced by polyethylene glycol-mediated transformation into *Arabidopsis* protoplasts for transient expression (23), and the accumulation of CryIC toxin was monitored by immunoblotting (Fig. 3*B*). In protoplasts carrying the bacterial gene (*b-cryIC*), no toxin was detectable, whereas cells transformed with the synthetic gene (*s-cryIC*) accumulated significant amounts of CryIC protein (Fig. 3*B*).

The *cryIC* genes were transferred into pPCV91, a transferred DNA-based transformation vector carrying a selectable hygromycin resistance (*hpt*) gene. In the dual gene expression cassette of pPCV91 (Fig. 3*A*), a synthetic phosphinothricine

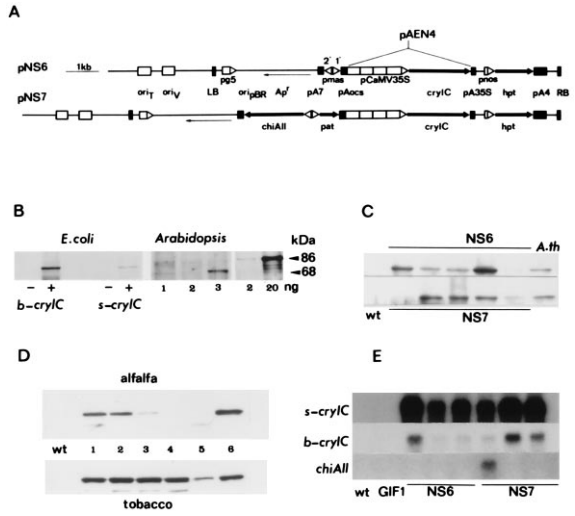


FIG. 3. Expression of *cryIC* genes in *E. coli*, *Arabidopsis*, alfalfa and tobacco. (*A*) Schematic map of plant transformation vectors. The *s-cryIC* gene was cloned in an optimized gene expression cassette in pNS6 between promoter (pCaMV35S) and polyadenylylation sequences (pA35S) from the 35S RNA gene of CaMV. The CaMV 35S promoter (18) contains four repeats of the upstream enhancer region (−90 to −418; marked by open boxes). The same CaMV 35S expression cassette is carried by pAEN4, a vector used for transient expression of *b-cryIC* and *s-cryIC* genes in *Arabidopsis* protoplasts. In addition to *s-cryIC*, vector pNS7 contains a phosphinothricine acetyltransferase gene (*pat*) under the control of a mannopine synthase (*mas*) 1′ promoter, and a chitinase AII (*chiAII*) gene driven by the *mas* 2′ promoter. The *s-cryIC* gene of pNS7 was exchanged for the bacterial *b-cryIC* gene in pGIF1. The structure of pGIF1 is otherwise identical with that of pNS7. ori_T and ori_V, Conjugational transfer and vegetative replication origins of plasmid RK2; LB and RB, the left and right 25 bp border repeats of the T-DNA, respectively; ori_{pBR}, replication origin of pBR322; Ap^R, bacterial ampicillin resistance gene; pg5, promoter of gene 5; pnos, nopaline synthase promoter; hpt, hygromycin phosphotransferase gene; pA4 and pA7, polyadenylylation signal sequences from the T-DNA encoded genes 4 and 7, respectively; pA_{ocs}, polyadenylylation signal sequence of the octopine synthase gene. Open arrowheads label plant promoters, and filled boxes mark plant polyadenylylation signal sequences. (*B*) Expression of *b-cryIC* and *s-cryIC* genes in *E. coli* and *Arabidopsis*. (*Left*) The *b-cryIC* and *s-cryIC* genes were cloned, respectively, in vectors pET-11a and pET-11d (24), and their expression in *E. coli* was monitored by immunoblotting with (+) or without (−) isopropyl β-thiogalactopyranoside (IPTG) induction, using a polyclonal anti-CryIC antibody. The lanes contain equal amounts of protein samples (15 μg) from *E. coli* extracts separated by SDS/PAGE. (*Right*) *Arabidopsis* protoplasts were transformed by polyethylene glycol-mediated DNA uptake with pAEN4 (1), and pAEN4-derived vectors carrying the *b-cryIC* (2) and *s-cryIC* (3) genes. After transient expression for 48 hr, samples containing 25 μg of soluble protein extract from protoplasts were separated by SDS/PAGE and subjected to immunoblotting. To estimate the amount of CryIC toxin in plant samples, purified CryIC protein of 86 kDa (carrying amino acid residues 1–756) was used as a standard (2 and 20 ng). (*C*) Screening for CryIC expression in alfalfa calli, carrying the transferred DNA of plant transformation vectors pNS6 and pNS7. Each lane contains 25 μg of soluble proteins from calli. For comparison, *Arabidopsis* protoplast extract (*A.th*), shown in lane 3 of *B*, was loaded as a standard, in addition to control protein extracts prepared from callus tissues of wild-type (wt) nontransformed alfalfa. (*D*) Screening for CryIC accumulation in leaf tissues of transgenic alfalfa and tobacco plants. Soluble proteins (50 μg) were prepared from NS6 (lanes 1 and 3–6) and NS7 (lane 2) alfalfa transformants, as well as from transgenic tobaccos carrying the NS7 *s-cryIC* gene construct (lower lanes 1–6). (*E*) Screening for transcripts of transgenes in leaves of soil-grown alfalfa plants carrying the transferred DNA of pGIF1, pNS6, and pNS7 vectors (three lanes each for NS6 and NS7 reflect three independent transgenic plants). Each lane in the three identical blots contains 20 μg of total RNA. The blots were hybridized, respectively, with *s-cryIC*, *b-cryIC*, and *chiAII* probes labeled to similar specific activity. Although several GIF1 transgenic plants expressing the *chiAII* gene were found during this screening (data not shown), no expression of the *b-cryIC* gene was detected in any of the GIF1 transformants. (The positive hybridizations with the *b-cryIC* probe are due to the partial homology between the synthetic and natural *cryIC* genes and the difference in the intensity of hybridizations with the *s-cryIC* and *b-cryIC* probes reflects differences between these *cryIC* sequences.)

acetyltransferase (*pat*) gene (20) was cloned downstream of the mannopine synthase (*mas*) 1′ promoter, to link the *cryIC* genes to a genetic marker allowing field-selection of transgenic plants by the herbicide BASTA. A chitinase AII (*chiAII*) gene from *Serratia marcescens* (21) was inserted downstream of the *mas* 2′ promoter, because previous studies (data not shown) indicated that chitinases may enhance the insecticidal activity of *Bt* toxins by destroying the chitinous peritrophic membrane of the insect midgut. The pPCV91 constructs, carrying the native, or synthetic, *cryIC* genes, either alone or in combination with the *pat* and *chiAII* genes, were introduced by *Agrobacterium*-mediated transformation into alfalfa and tobacco. From tobacco calli and somatic embryos of alfalfa selected on hygromycin, transformed shoots were regenerated. Transgenic plants derived from each transformation were assayed for the synthesis of CryIC toxin in leaves by immunoblotting and for *cryIC* gene expression using RNA hybridization. In calli or in plants carrying the bacterial *cryIC* gene (confirmed by DNA hybridization; data not shown), neither stable steady-state *cryIC* mRNA (Fig. 3*E*) nor toxin could be detected (data not shown). In contrast, transformed calli (Fig. 3*C*) as well as shoots carrying the synthetic gene (Fig. 3*D*), synthesized the CryIC toxin and accumulated significant amounts of steady-state *cryIC* mRNA (Fig. 3*E*). Shoots producing detectable amounts of CryIC toxin (0.01–0.2% of soluble leaf proteins) were vegetatively propagated and, if they carried the *pat* and *chiAII* genes, were further exposed to BASTA selection in the greenhouse and tested by RNA hybridization (Fig. 3*E*) using the corresponding genes as probes.

Transgenic Plants Expressing the *cryIC* Gene Are Resistant to the Egyptian Cotton Leafworm and Beet Armyworm. Transgenic alfalfa plants obtained by transformation with the pNS6 and pNS7 constructs (Fig. 3*A*) were tested for insect tolerance by feeding leaves to neonate larvae of the Egyptian cotton leafworm (*S. littoralis*). Fifteen of 27 NS6 transformants and 14 of 32 NS7 transformants produced 100% mortality of larvae (Fig. 4*A* and Table 1). Immunoblotting of leaf protein extracts showed that these plants produced 0.01–0.1% of total soluble protein as CryIC toxin in leaves (Fig. 3*D*). Leaves from these plants used in the diet of beet armyworms (*S. exigua*) also caused 100% mortality of larvae throughout their development (Fig. 4 *C* and *D* and Table 2). Screening of the NS7 transgenic alfalfa demonstrated that 15 out of 32 plants tested (47%) exhibited a high level of CryIC production (0.02–0.1% of total soluble protein), 2 plants (6%) had low toxin levels (less than 0.02%), and in 15 plants (47%) CryIC levels were below the detection limit of immunoblotting with 50 μg of soluble protein. NS6 transgenics consisted of 5/15 (33%) of high level, 7/15 (47%) low level, and 3/15 (20%) undetectable CryIC expressors.

Of 63 NS7 tobacco transformants, 42 (66.6%) were found to be resistant to 1.0% BASTA. Proper Mendelian segregation of BASTA and hygromycin resistance markers was confirmed after selfing 11 transgenic tobacco lines. Ten BASTA resistant plant stocks were assayed by immunoblotting and found to produce 0.1–0.2% of leaf soluble proteins as CryIC toxin (Fig. 3*D*). Three from these lines were used in bioassays with *S. exigua* and found to cause 100% mortality of larvae from different developmental stages.

To imitate field conditions, CryIC-expressing plants were infested with 15–20 larvae of the third to fourth instar stage in

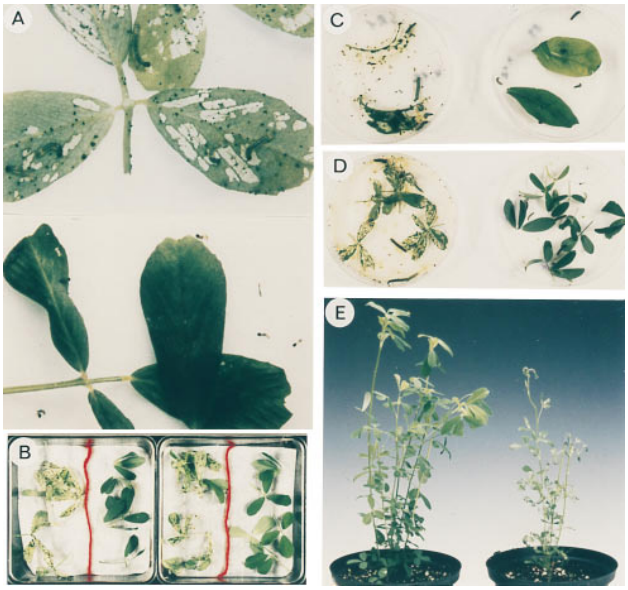


FIG. 4. Screening for *Spodoptera* resistance of transgenic plants. (*A*) Insecticidal assay with neonate larvae of *S. littoralis* reared for 2 days on leaves from nontransformed alfalfa (*M. sativa*, *Upper*) and NS7 transgenic (*Lower*) plants. (*B*) Free choice bioassays with leaves from wild-type and transgenic alfalfa plants. In the plate to the left, 10 larvae of *S. exigua* (third instar) were placed on the red line located between leaves of wild-type (*Left*) and NS7 transgenic (*Right*) alfalfa plants. In the plate to the right, the larvae were placed between leaves from wild-type (*Left*) and NS6 (*Right*; Fig. 3*D*, lane 6) transgenic alfalfas. For 5 days, the larvae failed to colonize leaves from the transgenic plants in both assays. (*C* and *D*) Leaves from tobacco (*C*) and alfalfa (*D*) plants were used for feeding of five fifth instar larvae of *S. exigua* for 10 hr. Petri dishes to the left in *C* and *D* contained leaves from nontransformed plants. Leaves shown in Petri dishes to the right in *C* and *D* were collected from a NS7 tobacco transgenic line producing 0.2% of soluble proteins as CryIC toxin (Fig. 3*D*, lane 2) and from a NS6 alfalfa transformant producing 0.1% of leaf proteins as CryIC toxin, respectively. (*E*) Transgenic NS7 (*Left*; Fig. 3*D*, lane 2) and nontransformed alfalfa (*Right*) plants were infested with 15 larvae of *S. exigua* (third to fourth instar stage) for 6 days.

the greenhouse. After 6 days, no viable insect escapes were detected, and the transgenic plants suffered barely detectable leaf damage; on average, less than 1% of the leaf area was affected (Fig. 4*E*). Infestation of a mixed population of wild-type and transgenic plants carrying the *s-cryIC* gene resulted in the devastation of wild-type plants, but yielded no apparent colonization by worms of the CryIC toxin-expressing plants in the population. Similar results were obtained by infesting detached leaves from these plants with larvae of *S. exigua* (Fig. 4*B*).

DISCUSSION

Spodoptera species are polyphagous cutworms and armyworms that may amplify to enormous numbers and devastate large agricultural areas (14). The widespread beet armyworm attacks rice, sugarbeet, alfalfa, cotton, corn, tobacco, tomato, potato, onions, peas, citrus, sunflower, and many grasses. The Egyptian cotton leafworm, a major pest in African and Mediterranean countries, favors fodder crops but also feeds on vegetables, industrial crops, medical plants, ornamentals, and trees. Young *Spodoptera* larvae may be controlled by pyrethroids, DDT, chlorinated hydrocarbons and organophosphorous insecticides (14, 28). However, because the eggs are laid on grassland, the efficiency of chemical insecticides, including methomyl and Pirate (AC303630), is rather limited (28). During the past few decades a considerable effort was therefore invested into the development of alternative insecticides to control armyworms.

Table 1. Control of *S. littoralis* (Egyptian cotton leafworm) by transgenic alfalfa plants

	Mortality of neonate larvae		
Construct	95–100%*	30–90%*	<30%*
NS6	15/27 (55.5%)	5/27 (18.5%)	7/27 (33.3%)
NS7	14/32 (43.8%)	5/32 (15.6%)	13/32 (40.6%)

From 60 NS7 transgenic alfalfa plants, 14 lines were found to be resistant to 0.1–0.2% BASTA. From these plants, nine lines displayed high levels (0.02–0.1%) of CryIC toxin production in leaves and caused 100% mortality of both *S. littoralis* and *S. exigua* larvae.

*The figures show the ratio between the numbers exhibiting the corresponding mortality rate to the total number of transgenic plants tested; in parenthesis, this fraction is expressed as a percentage.

Table 2. Control of *S. exigua* (beet armyworm) by transgenic alfalfa plants

	Mortality of larvae fed on leaves of plants with 0.02–0.1% CryIC toxin level		Time of scoring, days
Instar	NS6*	NS7*	
1	100% (1)	100% (3)	3
2–3	100% (5)	100% (7)	5
3–4	100% (2)	100% (3)	5
4–5–6	100% (3)	100% (2)	7

*The number of plants tested is in parentheses.

From the numerous insecticidal crystal proteins of *Bt*, CryIC was found to be the most active against *Spodoptera* (15). Other *Bt* toxins, such as CryIA(c), affect the beet armyworm only when produced in extraordinary high amounts in transgenic plants (7, 13) but do not provide protection (28). The engineering of CryIA toxin-producing crops prompted new research aiming to construct *Spodoptera*-specific CryIA toxins using recombinant *cryIA-cryIC* genes (29). As was done previously, we also attempted to express the bacterial *cryIC* gene in plants, using an optimized sequence context for enhancing its transcription and translation. Our failure to detect stable mRNA synthesized from the bacterial *cryIC* gene in transgenic plants supports the view that insect resistance cannot be achieved by expression of bacterial *cryI* sequences in plant cell nuclei.

Therefore, we developed a simple and economical gene synthesis method to construct large synthetic genes by ligation of oligonucleotide modules, using partial annealing with a single-stranded DNA template derived from a wild-type gene. The TDL-PCR method requires the synthesis of oligonucleotides comprising only one strand, in contrast to other technologies. This approach is free from the drawbacks of previous strategies for assembly of synthetic oligonucleotides, drawbacks such as multiple steps, a sensitivity to the secondary structure of oligonucleotides, complex effects of mispriming events, and polymerase fidelity problems with the increasing number of PCR steps. The gene assembly by ligation of oligonucleotides is preferable to assembly by the PCR approach, because no new errors are introduced to the assembled DNA. However, the ligation at conventional temperatures renders it very sensitive to secondary structures in DNA. Application of thermostable ligases may circumvent this problem (9). We used thermostable *Pfu* DNA ligase to perform thermal cycling for assembly, selection ("purification"), and ligation of full-length oligonucleotides as well as for linear amplification of the TDL product. Using the TDL-PCR method, it is not necessary to purify full-length oligonucleotides that may only represent 5–10% of the synthesis products with large linking numbers (e.g., over 80 nucleotides). Because of chemical phosphorylation, only full-length adjacent oligonucleotides, annealed in a sequence-specific order on the

template, can be ligated, because only they carry a 5′ phosphate. Thus, TDL by *Pfu* ligase automatically selects for and amplifies the designed synthetic gene segments. Avoidance of laborious procedures for the purification of long oligonucleotides gives the TDL-PCR method a significant advantage over other methods for gene synthesis. In a single TDL reaction, 5–8 oligonucleotides of 80–130 bases can be ligated and then converted to large double-stranded DNA segments by selective PCR amplification. In our method, PCR is used only in the final step, thus avoiding the assembly of oligonucleotides, in contrast to PCR-based methods for gene synthesis. Analysis of the observed errors showed that these originate from chemical oligonucleotide synthesis, a common problem with any gene synthesis technology, and that PCR did not introduce additional errors.

The TDL-PCR gene synthesis method allowed us to easily modify about 15% of the *cryIC* sequence and to remove cryptic polyadenylylation sites (27), potential splicing sequences, and other sequence motifs causing RNA instability, as well as to change the codon usage to fit plant codon preference and to optimize the translation initiation site according to the eukaryotic consensus (26). Comparison of the expression of native and synthetic *cryIC* genes in *E. coli* and *Arabidopsis* demonstrated the anticipated correlation between codon usage and translation. To ensure the production of CryIC toxin in plants, the synthetic *cryIC* sequence was placed in an optimized gene expression cassette to enhance its transcription by repeats of the enhancer elements of the CaMV 35S promoter (18) and to stimulate translation by linking it to an upstream untranslated leader of a plant RNA virus (19). Plant cells, expressing the synthetic gene transiently or stably, accumulated significant levels of *s-cryIC* mRNA and derived toxin protein, allowing the "molecular breeding" of *Spodoptera*-resistant plants that passed rigorous screening with insecticidal assays, immunoblotting, and RNA hybridizations. To facilitate the transfer of the *s-cryIC* gene to other alfalfa and tobacco cultivars by genetic crosses, the synthetic gene was combined with a BASTA herbicide resistance marker, allowing field selection of transgenic stocks. A *chiAII* gene was also expressed in the transgenic plants to test potential synergism between *Bt* toxin and chitinase. Insecticidal assays *in vitro*, as well as heavy infestations of greenhouse plants, showed that the expression of CryIC toxin in alfalfa and tobacco plants confers resistance to two *Spodoptera* species, the beet armyworm and the Egyptian cotton leafworm. A synergistic effect between chitinase AII and the CryIC toxin could have escaped our detection because toxin levels as low as 0.01% of total leaf protein were sufficient to give 100% mortality of the larvae used in our assays. The data described above suggest that it is worth to challenge the characterized tobacco and alfalfa plants with natural *Spodoptera* invasions in the field, as well as to introduce the synthetic *cryIC* gene constructs into other plants of economical importance.

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1. Schnepf, E. H. (1995) *Curr. Opin. Biotechnol.* **6,** 305–312.
2. Knowles, B. H. (1994) *Adv. Insect Physiol.* **24,** 275–308.
3. Grochulski, P., Masson, L., Borisova, S., Pusztai-Carey, M., Schwartz, J.-L., Brousseau, R. & Cygler, M. (1995) *J. Mol. Biol.* **254,** 447–464.
4. Koziel, M. G., Beland, G. L., Bowman, C., Carozzi, N. B., Crenshaw, R., Crossland, L., Dawson, J., Desai, N., Hill, M., Kadwell, S., Launis, K., Lewis, K., Maddox, D., McPherson, K., Meghij, M. R., Merlin, E., Rhodes, R., Warren, G. W., Wright, M. & Evola, S. V. (1993) *Bio/Technology* **11,** 194–200.
5. Fujimoto, H., Itoh, K., Yamamoto, M., Kyozuka, J. & Shimamoto, K. (1993) *Bio/Technology* **11,** 1151–1155.
6. Wünn, J., Klöti, A., Burkhardt, P. K., Biswas, G. C. G., Launis, K., Iglesias, V. A. & Potrykus, I. (1996) *Bio/Technology* **14,** 171–176.
7. Perlak, F. J., Deaton, R. W., Armstrong, T. A., Fuchs, R. L., Sims, S. R., Greeplate, J. T. & Fischhoff, D. A. (1990) *Bio/Technology* **8,** 939–943.
8. Perlak, F. J., Fuchs, R. L., Dean, D. A., McPherson, S. A. & Fischhoff, D. A. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 3324–3328.
9. Sutton, D. W., Havstad, P. K. & Kemp, J. D. (1992) *Transgenic Res.* **1,** 228–236.
10. Adang, M. J., Brody, M. S., Cardineau, G., Eagan, N., Roush, R. T., Shewmaker, C. K., Jones, A., Oakes, J. V. & McBridge, K. E. (1993) *Plant Mol. Biol.* **21,** 1131–1145.
11. Perlak, F. J., Stone, T. B., Muskopf, Y. M., Peterson, L. J., Parker, G. B., McPherson, S. A., Wyman J., Love, S., Reed, G., Biever, D. & Fischhoff, D. A. (1993) *Plant Mol. Biol.* **22,** 313–321.
12. van Aarssen, R., Soetaert, P., Stam, M., Doeckx, J., Gosselé, V., Reynaerts, A. & Cornelissen, M. (1995) *Plant Mol. Biol.* **28,** 513–524.
13. McBride, K. E., Svab, Z., Schaaf, D. E., Hogan, P. S., Stalker, D. M. & Maliga, P. (1995) *Bio/Technology* **13,** 362–365.
14. Hill, D. S. (1983) *Agricultural Insect Pests of the Tropics and Their Control* (Cambridge Univ. Press, Cambridge, U.K.).
15. Yamamoto, T. & Powell, G. (1993) in *Advanced Engineered Pesticides*, ed. Kim, L. (Dekker, New York), pp. 3–42.
16. Strizhov, N., Keller, M., Koncz-Kálmán, Z., Regev, A., Sneh, B., Schell, J., Koncz, C., & Zilberstein, A. (1996) *Mol. Gen. Genet.*, in press.
17. Koncz, C., Martini, N., Szabados, L., Hrouda, M., Bachmair, A. & Schell, J. (1994) in *Plant Molecular Biology Manual*, eds. Gevin, S. B. & Schilperoort, R. A. (Kluwer, Dordrecht, The Netherlands), Vol. B2, pp. 1–22.
18. Fang, R.-X., Nagy, F., Sivasubramaniam, S. & Chua, N.-H. (1989) *Plant Cell* **1,** 141–150.
19. Gallie, D. R. & Kado, C. I. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 129–132.
20. Strauch, E., Arnold, W., Alljah, R., Wohlleben, W., Pühler, A., Eckes, P., Donn, G., Ulmann, E., Hein, F. & Wengenmayer, F. (1988) Eur. Patent EP 275957B1.
21. Shapira, R., Ordentlich, A., Chet, I. & Oppenheim, A. (1989) *Phytopathology* **79,** 1246–1249.
22. D'Halluin, K., Botterman, J. & De Greef, W. (1990) *Crop Sci.* **30,** 866–871.
23. Mathur, J., Koncz, C. & Szabados, L. (1995) *Plant Cell Rep.* **14,** 221–226.
24. Studier, W., Rosenberg, A., Dunn, J. & Dubendorff, J. (1990) *Methods Enzymol.* **185,** 60–89.
25. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY).
26. Kozak, M. (1986) *Cell* **44,** 283–292.
27. Dean, C., Tamaki, S., Dunsmuir, P., Favreau, M., Katayama, C., Dooner, H. & Bedbrook, J. (1986) *Nucleic Acids Res.* **14,** 2229–2240.
28. Burris, E., Graves J. B., Leonard, B. R. & White, C. A. (1994) *Fla. Entomol.* **77,** 454–459.
29. van der Salm, T., Bosch, D., Honée, G., Feng, L., Musterman, E., Bakker, P., Stiekema, W. J. & Visser, B. (1994) *Plant Mol. Biol.* **26,** 51–59.