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a BamHI site, and a 3′ primer (5′-ATGATCTAGATGCAG-
TACCG-3′). The 3′ primer was complementary to an oligo-
nucleotide (5′-GTCAACTAACAAGGGAAGTTTATACG-
GACCCACGGCACTGGATAGATGATC-3′) at the 3′ end of block I that carried cryIC sequences with the HinII site and unrelated overhang sequences with an XhoI site. The oligonucleotide at the 3′ end of block II (5′-GATAAATCGAGGC-
GACCTAATCTGAATAGGATATCCATATTGGC-3′) added unique DNA sequences with an XhoI site to the cryIC sequences upstream of the HinII site and matched a PCR primer (5′-GATAAATCGAGCCGGACCT-
TA-3′). The 3′-terminal oligonucleotide in block II carried cryIC sequences extending to the BglII site and downstream overhang sequences with an XhoI site that were complementary to a PCR primer (5′-CTGTCTGAATTCAGAGGAC-3′). In the oligonucleotide located at the 5′ end of block III, an EcoRI site was added upstream of the BglII site of cryIC gene, fitting to a PCR primer (5′-CTGTCTGAATTCAGAGGAC-3′). The oligonucleotide at the 3′ end of block III carried a BamHI site, following the position of TAG stop codon in the pR1 phage-
mid, as well as adjacent unique sequences with a NotI site that were complementary to a PCR primer (5′-AGCATTCGGCC-
GCGGATCTC-3′).

TDL reactions were carried out at a template/oligonucle-
tide ratio of 1:200 (a total of 0.05 μM of template versus 10 
μM 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 
M MgCl₂/0.1% Nonidet P-40/0.5 mM rATP/1 mM DTT) and 4 units of Fru 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 fragment of rATP (0.5 mM) and 4 units of Fru ligase was added. Five microliters from the TDL reaction mix served as template for PCR amplification with 100 μM 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 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 BamHI–XbaI (block I), XhoI–XbaI (block II), and EcoRI–NotI (block III), then cloned in pBlue-
script SK(+) to verify their DNA sequences. The errors (three small deletions, one transversion, and one transversion) 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 NotI 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 HindIII site of pPCV720. Upstream of a BamHI 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 BamHI site, it carried a polyadenylation signal sequence derived from the CaMV 35S RNA gene. A BamHI site present in the mannopine synthase promoter (pmas) of pPCV720 was replaced by a NotI site using a Sall-Δ–NotI adaptor (5′-GATCTGGGCGGCGC-3′). The resulting vector, pPCV91, carried three plant gene expression cassettes with unique HindIII, NotI, and SalI cloning sites. To construct pNS6, the synthetic cryIC gene was cloned as a BamHI fragment downstream of the CaMV 35S promoter (see Fig. 3A). In pNS7, a synthetic pat gene, encoding a phosphonothri-
cone acetyltransferase (20), and a chiiA gene from Serratia marcescens (21) were inserted into the SalI and NotI sites located downstream of the mas 1 and 2 promoters, respect-
vately. A bacterial cryIC gene from Bt ssp. aizawai 7.29 (Gen-
Bank 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 (Medi-
cis 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 BamHI site of a pAE4 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 × 10⁸ 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 immuno-
blotting. 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, respect-
evously, 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 (Amer-
sham).

RNA (20 μg) samples isolated from leaves and petioles of alfalfa plants were separated on agarose-formaldehyde gels (25). BamHI fragments (1.9 kb), carrying either synthetic or bacterial cryIC sequences (see Fig. 1), and a NotI 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 beef army-
worm (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 transgen-
ic 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-term to C-terminal toxin 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 polyadenylation signals (27), 12 ATTGA 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 intro-
duced downstream of amino acid codon 630. The G+C

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**Agricultural Sciences: Strizhov et al.**

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Automated synthesis of 5'-phosphorylated oligonucleotides

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

30-60 TDL-cycles

Pfu ligase, ATP
melting (92°C, 1 min)
annealing and
ligation (52°C, 3 min)

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.

neous annealing. The synthetic cryIC sequences were converted to double-stranded DNA fragments and specifically amplified by PCR using short end-primersthat 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 HinclII- and BglII 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 erro-
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. 3E) nor toxin could be detected (data not shown). In contrast, transient expression in tobacco calli (Fig. 3C) as well as shoots carrying the synthetic gene (Fig. 3D), synthesized the CryIC toxin and accumulated significant amounts of steady-state cryIC mRNA (Fig. 3E). 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. 3E) 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. 3A) 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. 4A 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. 3D). Leaves from these plants used in the diet of armyworms (S. exigua) also caused 100% mortality of larvae throughout their development (Fig. 4C 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. 3D). 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 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.)
Left the plate to the right, the larvae were placed between leaves from nontransformed alfalfa (M. sativa) and transgenical alfalfa plants (third to fourth instar stage) for 6 days. 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. 3D, lane 2) and from a NS6 alfalfa transformant producing 0.1% of leaf proteins as CryIC toxin, respectively. (E) Transgenic NS7 (Left; Fig. 3D, lane 2) and nontransformed alfalfa (Right) plants were infested with 15 larvae of S. exigua (third to fourth instar stage) for 6 days.

**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.

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 mismising 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

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

<table>
<thead>
<tr>
<th>Construct</th>
<th>Mortality of neonate larvae</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>95–100%*</td>
</tr>
<tr>
<td>NS6</td>
<td>15/27 (55.5%)</td>
</tr>
<tr>
<td>NS7</td>
<td>14/32 (43.8%)</td>
</tr>
</tbody>
</table>

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**

<table>
<thead>
<tr>
<th>Instar</th>
<th>NS6*</th>
<th>NS7*</th>
<th>Time of scoring, days</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100% (1)</td>
<td>100% (3)</td>
<td>3</td>
</tr>
<tr>
<td>2–3</td>
<td>100% (5)</td>
<td>100% (7)</td>
<td>5</td>
</tr>
<tr>
<td>3–4</td>
<td>100% (2)</td>
<td>100% (3)</td>
<td>5</td>
</tr>
<tr>
<td>4–5–6</td>
<td>100% (3)</td>
<td>100% (2)</td>
<td>7</td>
</tr>
</tbody>
</table>

*The number of plants tested is in parentheses.
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 polyadenylation 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 stock. A chiAll 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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