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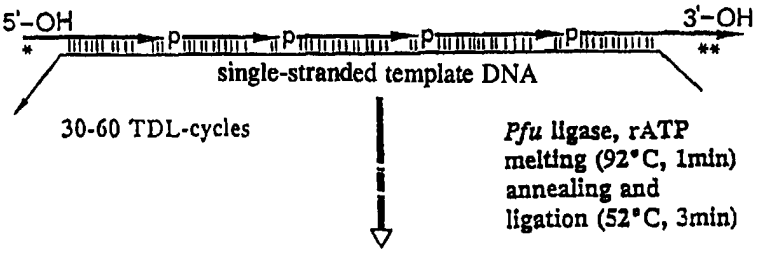
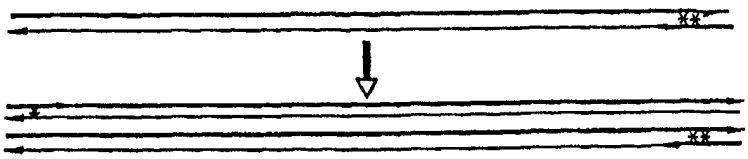
1: Automated synthesis of 5'-phosphorylated oligonucleotides

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



3: Selective PCR amplification of synthetic DNA strand



(57) Abstract

The present application relates to a synthetic version of a gene encoding the CrylCa toxin (formerly called CrylC) for expression in plants. The transgenic plants transformed with the synthetic gene express the insecticidal crystal protein toxin and become resistant to insects of the Spodopters species and the Mamestra species. Also disclosed are insecticidal formulations to control these insects.

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SYNTHETIC BACILLUS THURINGIENSIS GENE ENCODING CRYLCA (CRYLC) TOXIN

This application is a continuation-in-part of provisional application Serial No. 60/027,896, filed October 7, 1996, which is incorporated herein by reference.

FIELD OF THE INVENTION

The present invention relates to a synthetic version of a gene isolated from *Bacillus thuringiensis* (hereinafter "Bt" *or "B. thuringiensis"* encoding an insecticidal crystal protein designated CryIC, plants transformed with the gene, and the insecticidal crystal protein toxin expressed by the gene, all of which are used to control insects of the *Spodoptera* species as well as those of the *Mamestra* species.

BACKGROUND OF THE INVENTION

Insect infestation is responsible for millions of dollars of losses to commercially valuable agricultural crops each year. More than three billion dollars is spent worldwide annually to control insect pests. Traditionally, crops have been controlled from insect pests primarily through the use of toxic sprays. Unfortunately, residues of the sprays remaining on the fruits and vegetables have accumulated in human tissues, often with adverse effects, while at the same time many insects have become immune or resistant to the toxins. Additionally, the sprays often kill useful organisms, and precipitation runoff washes the toxins into streams and other bodies of water often killing fish.

Because of these and other disadvantages of using toxic sprays, alternative means of crop protection have been developed. One approach is the use of biological pesticides. One such agent is the bacteria *B. thuringiensis* which has been very effective against a

variety of caterpillars and worms. This bacteria has been traditionally sold in the form of a dust containing millions of spores. When the spores are sprayed on plants, they are harmless to humans and animals other than the target insect. During its sporulation cycle, *Bt* produces proteins toxic to certain pests in crystal form known as crystal delta-endotoxins. When the insect ingests any plant tissue with *Bt* spores on it, the bacteria quickly becomes active and multiplies within the insect's digestive tract, soon paralyzing the gut. The insect stops feeding within two or three hours.

The delta-endotoxin are encoded by crystal protein ("*cry*") genes. Thus far, over 100 Cry proteins were identified and classified according to their sequence homology and insect specificity (reviewed in *Höfte* and *Whiteley*, 1989; *Aronson 1993*, *Schnepf* 1995). The *cry* genes have been divided into six classes and several subclasses based on structural similarities and insecticidal specificity. The major classes are as follows:

Class	Insect Specificity
cryI	Lepidoptera (butterflies, moths)
cryII	Lepidoptera and Diptera (flies, mosquitos)
cryIII	Coleoptera (beetles, weevils)
cryIV	Diptera (flies, mosquitos)
cryV	Coleoptera and Lepidoptera
cryVI	Nematode (roundworms)

With particular regard to the lepidoptera - specific crystal proteins ($_{cryI}$), to which the present invention is directed, six subclasses having different gene types have been identified. Subclasses of the *cryI* genes include the following: *cryIA(a)*, *cryIA(b)*, *cryIA(c)*, *cryIB*, *cryIC*, and *cryID*. CryIC endotoxin is the most active *B. thuringiensis* crystal protein against the *Spodoptera* species which includes the following pests: *S.*

littoralis, *S. exempta*, *S. exigua*, *S. frugiperda*, *S. litura* and others.

Unfortunately, production of the bacterial spores for commercial use is limited and the protective effect is short-lived. Accordingly, plant molecular biologists have developed transgenic plants that express the *Bt* toxin within their cells and tissues which have been effective against pests which feed on the leaves of the plant. For example, U.S. Patent No. 5,187,091 to *Donovan et al.* describes incorporating into a plant a *cryIIIC* gene thereby rendering the plant more resistant to insect attack. Additionally, tobacco and tomato plants expressing the *Bt* toxin gene reportedly have killed larvae of tobacco hornworms. However, the wild-type crystal gene is poorly expressed in transgenic plants. Hence, protection is not attained against less sensitive, but agronomically important, insect pests like the cotton bullworm. *(Watson et al.* Recombinant DNA, 2d ed. 1992). The expression of the full-length lepidopteran specific *Bt* gene (*cryI* in particular has been reported to be unsuccessful in expressing insecticide in some plants. (Vaeck et al., 1987)

To increase expression in plants, truncated and synthetic genes containing codons preferred in plants have been successfully employed.

U.S. Patent No. 5,380,831 to *Adang et al.* discloses a synthetic *B. thuringiensis* gene designed to be expressed in plants at a level higher than naturally occurring *Bt* genes. The gene utilizes codons preferred in plants. The modifications described include the elimination of CUUCGG hairpins and plant polyadenylation signals and modifying the A+T content to that found in plants.

U.S. Patent No. 5,500,365 to *Fischoff et al.* discloses synthetic plant genes which encode insecticidal proteins of *Bt* for plant transformation wherein the genes express their protein product at higher levels than the

wild-type genes. In particular, they removed regions with many consecutive A+T bases or G+C bases as well as ATTA sequences and putative polyadenylation signals, and the condon usage was changed according to plant preferences.

The insecticidal spectrum of *Bt* thus far expressed in transgenic plants is limited. Genes encoding the processed forms of CryIA(a), (b) and (c) have been expressed in plant-associated bacteria and transgenic plants to control major insect pests of maize, rice, cotton, tomato, potato and tobacco. Nonetheless, insects of the *Spodoptera* species, which cause severe agricultural damage, have thus far escaped efficient control because of problems preventing a high level expression of CryIC toxins in transgenic plants. 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 feed on over 40 different plant families world-wide, including at least 87 species of economic importance. Armyworms, most of which fall within the genus *Spodoptera*, march in swarms from field to field devastatingly defoliating entire crops. In the United States corn, sorghum and peanut are crops upon which fall armyworm *(Spodoptera frugiperda)*, infestations often reach devastating levels. In one year, for example, losses in the state of Georgia alone were estimated at over 20 million dollars. Corn yield losses attributed to the fall armyworm for the United States have been estimated at 2% annually. In the southeastern United States, *S. frugiperda*, is a major pest of corn, sorghum and peanut, causing more than $60 million in damages per year. From the various insecticidal crystal proteins of *B. thuringiensis* expressed in transgenic plants that have been disclosed in prior art none showed activity required for plant protection against *Spodoptera* species. Moreover, no significant differences in leaf

area consumed, mortality or pupal weights of *S. exigua* larvae were detected between transgenic *B. thuringiensis* Monsanto cotton line and non-transformed plants *(Burris et al.,* 1994).

The Spodoptera species are polyphagous cutworms and armyworms, that may amplify to enormous numbers and devastate huge agricultural areas. The wide-spread beet armyworm *S. exigua* attacks rice, sugarbeet, alfalfa, cotton, corn, tobacco, tomato, potato, onions, peas, citrus, sunflower, and many grasses. The Egyptian cotton leafworm *S. littoralis,* a major pest in African and Mediterranean countries, favors fodder crops, such as alfalfa and clover, but also feeds on may vegetables, industrial crops, medical plants, ornamentals, and trees. Young *Spodoptera* larvae may be controlled by pyrethroids, DDT, chlorinated hydrocarbons and organophosphorus insecticides. However, because the eggs are laid on grassland, the efficiency of chemical insecticides, including the most efficient compounds methomyl and Pirate (AC303630), is rather limited. During the last decades a considerable effort was therefore invested into the development of safe insecticides to control armyworms in an environmentally friendly fashion.

Despite the significant damage caused by *Spodoptera* insects, safe and efficient pest control through the genetic engineering of plants is lacking because of the difficulty of achieving a high level of expression of *CryIC* toxin in transgenic plants. It would therefore be most desirable to have a gene encoding *CryIC* toxin that can be expressed in transgenic plants thereby safeguarding them against *Spodoptera* pests in an effective yet environmentally friendly manner.

SUMMARY OF THE INVENTION

The present invention relates to a synthetic Bt gene that expresses CryIC delta-endotoxins against *Spodoptera* insects when expressed in plants transfected by the gene.

More specifically, it comprises a chemically synthesized gene coding for a truncated CryIC protoxin of 630 amino acids which has been expressed in alfalfa, tobacco, and potato plants and has proven to provide resistance to *S. littoralis and S. exigua*.

To improve the engineering of CryIC toxins, the gene according to the present invention establishes a consensus CryIC sequence within the boundaries of the entomocidal fragment of CryIC toxin that confers resistance to midgut proteases and larvae of *Spodoptera littoralis*. 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 of 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. Engineering of insects 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.

Various features of the natural *Bt* genes differ from those of plants and heterologous genes expressed in plants. *Bt* genes are rich in adenine (A) and thymine T (more than 62%) while plant exons have about 45% - 55% A+T content. Fortuitous plant processing signals present in *Bt* genes drastically diminish the level of their expression in plant cells. Efficient transcription of the synthetic *cryIC* gene according to the present invention in plant cell nuclei was achieved by the removal of AT rich sequences that may cause mRNA instability or aberrant splicing, 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. In addition, the sequence context around the translation start was modified to conform to the eukaryotic consensus.

Synthesis of the synthetic gene herein was accomplished using a unique method "TDL-PCR" described herein and in more detail in our co-pending U.S. application which is incorporated herein by reference.

The synthetic gene according to the present invention may be employed to transform most plants thereby protecting them against pests which are members of the *Spodoptera* genus (Lepidoptera, Noctuidae) which feed on over 40 different plant families world-wide, including at least 87 species of economic importance (Hill, 1983). For example, the widespread beet armyworm *S. exigua* attacks rice, sugarbeet, alfalfa, cotton, corn, tobacco, tomato, potato, onions, peas, citrus, sunflower, and many grasses. The Egyptian cotton leafworm *S. littoralis*, a major pest in African and Mediterranean countries, favors fodder crops, such as alfalfa and clover, but also feeds on may vegetables, industrial crops, medical plants, ornamentals and trees.

Additionally, the CryIC toxin that is expressed by transgenic plants according to the present invention can be collected and used, for example, as an insecticidal spray due to the fact that the protein is water soluble. CryIC toxins produced by bacteria, in contrast, are water insoluble rendering them undesirable for a variety of industrial and agricultural applications.

With the foregoing and other objects, advantages and features of the invention that will become hereinafter apparent, the nature of the invention may be more clearly understood by reference to the following detailed description of the invention, the figures, and the appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a schematic plan for the gene synthesis method according to the present invention.

Fig. 2 shows the nucleotide sequence of the synthetic *cryIC* gene. Where different, the native

(bacterial) sequence is shown above. The amino acid sequence is shown below.

Fig. 3a is a schematic map of plant transformation vectors.

Fig. 3b is a photograph of Western blots showing expression of *b-cryIC and s-cryIC* genes in *E. coli and Arabidopsis*.

Fig. 3c is a photograph of Western blots showing screening for *cryIC* expression in alfalfa.

Fig. 3d is a photograph of Western blots showing screening for cryIC accumulation in leaf tissue of transgenic alfalfa and tobacco plants.

Fig. 3e is a photograph of a Northern blot showing screening for transcripts of transgenes in leaves of soil-grown alfalfa plants.

Fig. 3f is a Western blot analysis of leaf protein extracts from transgenic potato plants expressing the *cryIC* gene. 10 ng of truncated CryIC produced in *E. coli* (positive control), total proteins (50 μg) from plant var. Desiree (negative control) and transformed plant number 1,3,5 and 6 containing 2,2,1 and 1 copies of the cryIC gene respectively, were electrophoresed on 12% polyacrylamide gels, then transferred to PVDF membrane. The blot was incubated with rabbit-anti CryIC, and then with horseradish peroxidase conjugated to anti-rabbit immunoglobulin. Enzymatic visualization of immunoreactive CryIC was then carried out.

Fig. 3g is a Northern blot analysis of *cryIC* gene transcripts in transgenic potato plants. Total RNA (15 μg) extracted from untransformed Desiree plant and transgenic plants ARI (1,2,5 and 6) resulted from transformation with the binary vector pAR1, were electrophoresed with glyoxal in 1 % agarose TBE gels, then transferred onto nylon membrane and probed with ^{32}P labeled $_{cryIC}$ gene. The number of introduced *cry* copies per plant is indicated below.

Fig. 4 is a photograph showing screening for *Spodoptera* resistance in transgenic plants.

(A) neonate larvae of *S. littoralis* reared on transgenic (bottom) and nontransformed (top) alfalfa *(M. sativa)* plants.

(B) "free choice" bioassays with leaves from transgenic (right) and nontransgenic (left) alfalfa plants and larvae of *S. exigua* (3rd instar).

(C-D) leaf (C-from tobacco; D-from alfalfa) bioassays with 5th instar larvae of *S. exigua* reared on leaves taken from transgenic (right) and nontransgenic (left) plants.

(E) bioassays with alfalfa soil grown transgenic (left) and non-transgenic (right) plants and larvae of *S. exigua*.

Fig. 5 is a Bioassay of transformed potato plants (Desiree). *Spodoptera littoralis* larvae of the 2nd to 4th instars were fed on leaves of Desiree (control) and primary transformants. 1-plant No. AR1(2), 2-plant No. AR1(1), 3-plant No. AR1(3), 4-plant No. ARI(5), 5-plant No. AR1(6). Leaves were photographed 48h after being exposed to the larvae.

Fig. 6 is a Southern blot analysis which confirms the integration of the *cryIC* gene into plant genome. Plant DNA (20,μg) was digested with the restriction enzyme EcoRI in panel A and XbaI in panel B and electrophoresed on 0.8 % agarose TAE gels. DNA was then transferred onto nylon membranes and probed with ^{32}P labeled *hph* (a), *cry*IC(b) and with *Amp* resistance gene (c). Lane I - control Desiree plant, lanes 2-8 transformed potato plants No. 1,2,3,5,6,7,8 carrying 2, > 8, 2,1,1,3 and 1 copies of the *cry*IC gene respectively. Partial digestion with XbaI (which is sensitive to methylation) of plant DNA extracted from potato plant carrying at least 8 copies of the synthetic cryIC gene (panel B, lane 3), suggests possible involvement of plant hyper-methylation.

Fig. 7 is a Western blot analysis of total proteins extracted from the transgenic plant AR1(1) and incubated *in vitro* with gut proteases from 4th instar larvae. Similar AR1(1) protein samples were subjected to sequential denaturation and renaturation (D/R) which completely abolished their resistance to proteolysis (last mixed with long *E. coli* produced CryIC (630aa) were incubated with gut proteases. No resistance of bacterial CryIC to proteases was observed. Following incubation the protein samples were separated on 12% SDS-acrylamide gel, blotted onto PVDF membrane and probed with ani-CryIC polyclonal antibodies.

From left to right:

Lane 1 AR1(1) proteins, no gut proteases.

Lane 2-4 AR1(1) proteins incubated with gut proteases (o.2μg proteins, depicted as enzIV) for 2, 10 and 20 min. respectively.

Lane 5 Proteins of non-transformed Desiree plant.

Lane 6 Proteins of non-transformed Desiree plant mixed with long *E. coli* produced CryIC (630aa).

Lane 7-9 as in 6 but incubated with gut proteases for 1, 10 and 20 min. respectively.

Lane 10 AR1(1) proteins after denaturation and renaturation as detailed below.

Lane 11-13 AR1(1) proteins subjected to 6M Guanidinium hydrochloride (denaturation), dialyzed against 50mM Tris-HCI, pH8 over-night (renaturation) and then incubated with gut proteases for 2, 10 and 20 min. respectively.

DETAILED DESCRIPTION OF THE INVENTION

The process for gene synthesis is described in detail in Example I and is described in even greater detail in our co-pending U.S. application serial number _____ titled "Gene Synthesis Method" filed on

December 20, 1996, which is incorporated herein by reference. In short, chemically synthesized and phosphorylated oligonucleotides of the gene to be created are assembled on a single-stranded partially homologous template DNA derived from the natural or wild-type gene. After annealing, the nicks between adjacent oligonucleotides are closed by a thermostable DNA ligase followed by repeated cycles of melting, annealing, and ligation. This template directed ligation ("TDL") results in a new single-stranded synthetic DNA product which is subsequently amplified and isolated from the wild type-template strand by the polymerase chain reaction (PCR) with short flanking primers that are complementary only to the new synthetic strand. These PCR end-primers contain suitable restriction cleavage sites for cloning of the synthetic double-stranded DNA fragments. This process is illustrated schematically in Fig. 1.

Although the gene according to the present invention was used to successfully transform alfalfa, *Arabidopsis*, tobacco and potato plants, it can be used to transform any other dicot in a similar manner so as to render the plant resistant to insect attack. Genetic engineering of plants with the *cryIC* gene (Fig.2) may be accomplished by introducing a vector containing the gene into the plant cells using one of a variety of vectors known to those in the plant genetic engineering art. The synthetic cryIC gene according to the present invention may be delivered into the plant cells by *Agrobacterium* mediated transformation, by microinjection, by bombardment with DNA-coated microparticles, by PEG medicated transformation, by electroporation and by other techniques known by those skilled in plant genetic engineering.

The CryIC δ-endotoxin, and transgenic plants which express this insecticide, may be employed to safeguard against all members of the *Spodoptera* species. Important

Spodoptera pests include *S. exigua* (Beet armyworm), *S. litura* (Rice cutworm, Common cutworm), *S.maurita* (Paddy armyworm), *S. eridania* (Southern armyworm), *S. praefica* (Western yellow-striped armyworm), *S. ornithogalli* (Cotton cutworm), and others. The toxin according to the present invention is also effective against species of *Mamestra genus,* including *M.brassica* (Cabbage mo__, *M.configurata* (Bertha armyworm), *M.illoba* (Mulberry Caterpiller), *M. persicariae* (Beet Caterpiller) and others. The effective CryIC LC_{50} doses for *M.brassica* were reported at levels even 5 fold lower than those required for *S.littoralis* (Höfte, Whitely, 1989). *M.brassica* is a serious pest on many crops, mainly *Brassica* crops, totally polyphagous, abundant and widespread. *M. configurata* is an important economic pest on oil seed crops such as canola, *B.napus and B.rapae* in Canada and the United States.

The present inventors have confirmed the insecticidal activity of CryICa5 against *M. Brassica* and *M. Configurata*. The crystal protein was used in feeding assays on potato tuber slices and provided efficient control.

In other experiments, the crystal protein showed activity against *Phthorimaea operculella*. Furthermore, *P. operculella* was controlled also by feedings with tuber slices from transgenic plants which expressed the truncated synthetic CryIC gene of the invention.

Although CryIC δ-endotoxin is the most active *Bt* toxin against *Spodoptera and Mamestra* species, it has insecticidal activity towards other important pests of Lepidoptera order, such as *Trichoplasia ni* (Cabbage semilooper), *Plutella xylostella* (Diamondback moth) *Pieris brassica* (Large white butterfly), *Pieris rapae* (Small white butterfly) with the LC*50* doses, that are comparable or even lower of those required for protection against *Spodoptera* insects. Therefore, synthetic *cryIC* gene of present invention can be used not only as a

monotransgene, but it can also be included in various strategies with multiple *Bt* genes in order to fight or to avoid an appearance of *Bt* resistant insect pests.

In addition to the activity towards Lepideptera, the CryIC δ-endotoxin is toxic to the larvae of several dipteran insects, such as *Aedes aegypti, Anopheles gambia, Culex quinquefasciatus* (Smith et al., 1996). This fact opens a possibility to use the synthetic *cryIC* gene for creation of transgenic mammals in order to protect cattle and other suffering animals from dipteran vectors of various diseases as well as to protect livestock from irritating attacks of swarm of midges to increase, for example, milk or meat production.

Bt δ-endotoxins are accumulated in bacteria as insoluble inclusions, which upon ingestion by insect larvae must be activated by midgut proteases. Truncated Bt δ-protoxins are produced in *E. coli* as insoluble inclusion bodies, consisting of misfolded proteins, that in turn greatly reduces toxicity. However, the truncated CryIC produced in transgenic plants expressing the synthetic *cry*IC gene according to the present invention is highly soluble which renders it useful in a variety of industrial and agricultural applications. In transgenic *Arabidopsis,* containing the synthetic cryIC gene of the present invention, *cry*IC protein was accumulated up to 1% of total soluble protein, i.e., 25 ng per microliter in contrast to the solubility of the truncated CryIC produced in *E. coli* (0.8 ng per microliter). Whole amount of CryIC protein is deposited in a soluble fraction of the plant cell. The fact that the plant produced CryIC δ-endotoxin is soluble permits its use as a new product exploiting its solubility properties (e.g., a water based spray). The soluble CryIC protein produced by transgenic plants may be employed in insecticidal formulations either in an isolated form or with an agriculturally acceptable carrier that are well known to those skilled in insecticide formulation.

One disadvantage of microbial *Bt* formulations is a high price of the production requiring the marginally economic use of fermenters and media for bacterial growth. However, transgenic plants with synthetic *cry*IC according to the present invention, for examples alfalfa, are free from these limitations. Insect self-protected plant material can be collected during several years by cutting plants in fields. Due to its water solubility, the plant produced CryIC insecticide can be easily extracted from the collected plant material.

EXAMPLES:

The following Examples are provided to illustrate the practice of the invention and are not intended to limit the scope thereof.

EXAMPLE I--Gene Synthesis

Gene Construction

Figure 2 shows the nucleotide sequence of the synthetic *cry*IC gene (*s-cryIC*). Nucleotides of the bacterial *cry*IC *sequence* (*b-cry*IC) exchanged in the synthetic gene are shown in the upper lanes. The nucleotide sequence of the *s-cryIC* coding region for 630 codons starts with an ATG codon in a sequence context fitting the eukaryotic consensus 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. *Hinc*II and BglII cleavage sites used for the assembly of three TDL-PCR blocks are indicated by boxes above the sequences. The amino acid sequence of the truncated CryIC δ-endotoxin is displayed in single letter code below the *s-cryIC* sequence.

The designed DNA sequence of the *s-cryIC* gene (Fig. 2) was divided into three blocks separated by *Hinc*II and *Bgl*II cleavage sites. The *BamHI-HincII* block-I was constructed from eight, the *Hinc*II - *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 *B. thuringiensis cry*IC gene (Fig. 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 a *Bam*HI site and a 3'-primer (5'-ATGATCTAGATGCAGTAGCG-3'). The 3'-primer was complementary to an oligonucleotide (5'-GTCAACTAACAAGGGAAGTTTATACGGACCCACGCT ACTGCATCTAGATCAT-3') at the 3'-end of block-I, that carried *cry*IC sequences with the *HincII* site, and unrelated overhang sequences with an *Xba*I site. The oligonucleotide at the 5'-end of block-II (5'-G A T A A C T C G A G C G A G C C T A A A C T A T G A C A A T A G G AGATATCCAATTCAGCCAGTTG-3') added unique DNA sequences with an *Xho*I site to *the cryIC* sequences upstream of the *Hinc*II site and matched a PCR primer (5'-GATAACTCGAGCGAGCCTA-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 to the *Bgl*II site of *cryIC* gene, fitting to a PCR primer (5'-CTGTCTGAATICAAAGATC-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'-AGCATGCGGCCGCGGATCC-3').

TDL Technique

Template directed ligation (TDL) reactions were carried out at a template to oligonucleotide ratio 1:200 (a total of 0.05 pM of template versus 10 pM of each oligonucleotide) in a final volume of 50µl using a reaction buffer (20 mM Tris.HCl (pH 7.5), 20 mM KCl, 10 mM $MgCI_2$ 0.1% NP-40, 0.5 mM rATP, 1 mM DTT)-and 4 U *Pfu* DNA ligase (Stratagene), or any other similar thermostable DNA ligase.

Thirty cycles of TDL reactions were used to obtain a desirable amount of a TDL product. The temperature range during melting step is between 90 to 98°C with a preferable temperature of 92°, with 1 minute of required step time. Annealing and ligation were performed at a temperature range of 45 to 60°C with a preferable temperature of 52°C during required step time from 3 to 10 minutes. Melting step was followed by annealing and ligation step to obtain a TDL cycle which was repeated at least 30 times. To increase the number of TDL cycles for every additional 30 cycles a new portion of rATP (0.5 mM and 4 U of *Pfu* ligase was added. Temperature cycling during TDL step was done on a Perkin-Elmer thermal cycler (Norwalk, CT).

PCR Selective Amplification of Synthetic TDL-PCR Blocks

5 µl from the TDL reaction mix served as template for PCR amplification with 100 pM of primers, 250 µM dNTP and 2.5 U Ampli-Taq or any other similar thermostable DNA polymerase such as UlTma (Perkin-Elmer) polymerase in 100 µl buffer (10 mM Tris.HCl (pH 9.0), 50 mM KCl and 0.1 % Triton- X100), using 30 cycles at 92°C for 1 min, 45°C for 1 min, and at 72°C for 1.5 minutes, with final extension for 10 minutes, at 72° C. PCR amplifications were performed on a Perkin-Elmer (Norwalk, CT) thermo

cycler. The amplified DNA fragments were gel purified, digested with *Bam*HI-*Xba*I (block-I), *Xho*I-*Xba*I (block-II), and *EcoRI-Not*I (block-III), then cloned in pBluescript SK + to verify their DNA sequences.

Design of Synthetic *cry*IC Gene Aimed to be Transferred to Plants

a) Identification of the minimal entomocidal fragment of *Spodoptera* specific *Bt* toxin CryIC. From prior art it is known that expression of the truncated *Bt* genes in transgenic plants was superior compared to the full-length genes of protoxins. Moreover, identification of the minimal toxic fragment of a protoxin would significantly reduce efforts and costs involved in construction of a synthetic gene. In CryIA proteins the tryptic fragment, which is still responsible for toxicity, composes a half of the protoxin. To improve the production of recombinant CryIC toxins, we mapped the minimal toxic fragment of the protoxin by testing of various truncated proteins for the stability and protease sensitivity to trypsin and *Spodoptera* midgut protease as well as by the measurement of the insecticidal activity to *S. littoralis* larvae. In contrast to previous data, the boundaries of trypsin-resistant CryIC core toxin were Mapped to amino acid residues I28 and R627. Proteolysis of the truncated CryIC proteins showed that *Spodoptera* midgut proteases may further shorten the C-terminus of CryIC toxin to residue A615. However, C-terminal truncation of CryIC to residue L614, and a mutation causing amino acid replacement I610T abolished the insecticidal activity of CryIC toxin to *S. littoralis* larvae, as well as its resistance to trypsin and *Spodoptera* midgut proteases. Because no CryIC toxin carrying a proteolytically processed N-terminus could be stably expressed in bacteria, our data indicate that in contrast to other CryIC proteins an entomocidal fragment located between amino acid positions 1 and 627 is

required for stable production of recombinant CryIC toxins.

b) Establishment of the CryICa5 protein sequence. Design of the synthetic *cryIC* gene was based on the sequence of the corresponding wild-type gene *(cryICa5,* EMBL X96682), which we established after sequencing of three independent *cryIC* genes isolated from three different *B. thuringiensis* strains K26-21, MR1-37 (new isolates, collected from the soil samples in Kenya and Israel, respectively) and subsp. *aizawai* 7.29, all selected from high insecticidal activity against larvae of the *S. littoralis*. We have found that all the three strains contain the identical *cryIC* gene sequence, which has certain discrepancies with all the cryIC sequences known in prior art *(cryICa1* - Honee et al.; *cryICa2* - Sanchis et al.; *cryICa3* - U.S. Patent 5,246,852, 1993; *cryICa4* 0400246, 1995). Moreover, in fact, the *cryICa5* sequence represents, a consensus of all 12 known *cryIC* genes.

Corresponding sequence of CryICa5 protein differs by amino acid replacement A124E, A294R, and H453D from the CryICa1, by a T450Q exchanges from the CryICa3, and by the A124E from the CryICa4. Similarly, multiple sequence shifts resulting in N366I, V386G and 376WPAPPEN382 to 376CQRHHFN382 amino acid replacements were found in the previously published *CryIC* sequence from Subsp. aizawai 7.29 (Sahchis et al, 1989). The occurrence of glutamate in position 124 and glutamine in position 405 were clearly due to previous errors, since A124 and T405 were found to be conserved in all CryIC proteins. Similarly, sequence variations detected between Positions 366 and 386 of the *CryIC* sequence from Subsp. aizawai 7.29 could safely be excluded because they would wither create a new tryptic cleavage site, such as the R378 residue, or affect the activity and insect specificity of the toxin, such as the W376C replacement and the 374QPWP377 motive that are located in a surface exposed loop of the

variable toxin domain II. Therefore, we believe that the *CryIC* sequence, known in prior art, contain critical errors with negative consequences either for function or stability of *CryIC* protein, had the protein a corresponding synthetic gene designed on the basis of the wild-type DNA sequences known in prior art. Modifications of the synthetic *CryIC* gene (*s-Cry*IC) sequence of the present invention did not alter the amino acid sequence of the minimal toxic fragment of the CryICa5 protoxin, containing N-terminal fragment with the length of 630 amino acid residues.

The synthetic *cryIC* gene coding for an N-terminal protoxin fragment of 630 amino acids was designed (Fig. 2) by exchanging 286 bp of the bacterial *cry*IC sequence (EMBL 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, 12 ATTTA motifs, 68 sequence blocks with 6 or more consecutive A/T's, and all motifs containing 5 or more G+C or A+T nucleotides. Sequences around the translation initiation site were changed to conform to the eukaryotic consensus sequence, and a TAG stop codon was introduced downstream of amino acid codon 630. The G+C content of the *cryIC* gene was thus increased from 36.6% to 44.8%. The *s-cry*IC gene was synthesized from oligonucleotides of 70-130 bases that were chemically phosphorylated at their 5'-ends. Since 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 5-8 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 template directed ligation (TDL, Fig. 1) method 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 ligation 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 *Hinc*II and *Bgl*II digested DNA fragments, and cloned in pBluescript.

With further reference to the Figures relating to this Example, Fig. 2 shows the 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 and terminates at a TAG stop codon. Vertical black arrows above the *s-cryIC* sequence indicate the boundaries of adjacent synthetic oligonucleotides used for TDL-PCR gene synthesis. *Hinc*II and *Bgl*II cleavage sites used for the assembly of three TDL-PCR blocks are framed.

It will be understood by an artisan of average skill in the art that variations of the gene or of the toxin domain protein sequence can be created by well known molecular biology techniques. Biological assays in line with assays described herein allow for testing of the

variants to determine bio-suppression of insects. It is therefore to be understood that functional variants of the synthetic gene or protein are within the scope of the invention.

For example a variant gene can be produced by site-directed mutagenesis, whereby individual nucleotides are replaced, or short stretches of nucleotides are added or deleted in a manner that would not change the coding frame of the remaining sequence. Expression of the variant gene would produce a variant protein sequence. changes in the amino acid sequence would be particularly tolerated if these changes comprise conservative substitutions of amino acids. For example, leucine and isoleucine can be substituted for each other and would be considered a conservative amino acid substitution which would not be expected to substantially affect the folding, toxicity, or insect range of the toxin. conservative amino acid substitutions are well known in the art.

TABLE 1

Summary of Changes Introduced in the Truncated Synthetic *cryIC* Gene (*s-cryIC*) Compared to the Natural Counterpart (*n-cryIC*)

	s-cryIC	*n-cryIC*
G+C content	44.8% (exon like)	36.6% (intron like)
Bases different from wild type	285 of 1890 (15.1%)	-
Codons different from wild type	249 of 630 (39.4%)	-
Potential plant polyadenylation sequences (*Dean et al.*, 1986)	-	21
ATTTA sequences	-	12
A+T rich regions (< 6 Consecutive A and/or T)	-	68

All codons rarely used in plants and present in the wild type *cryIC* were substituted by the most preferred codons in alfalfa and dicots plants.

G+C runs of 5 or more and A+T runs of 5 or more were avoided in the synthetic *cryIC*.

The sequences upstream of the translation initiation site was changed according to the eukaryotic consensus sequences.

EXAMPLE II

Plant gene expression constructs and transformation of alfalfa and tobacco.

The plant expression vector pPCV91 was constructed by modification of pPCV720. A *NotI* site in the RK2-domain was eliminated by filling in with DNA polymerase Klenow fragment, and a CaMV35S promoter with four repeats of the enhancer domain (-90 to -418), was introduced into the *Hind*III 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 (TMV) RNA, whereas downstream of the *Bam*HI site it carried a polyadenylation signal sequence derived from the CaMV 35S RNA gene. A *Bam*HI site present in the mannopine synthase dual promoter (pmas) of pPCV720 was

replaced by a *NotI* site using a *Sau3A-NotI* 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 CaMV35S promoter. In pNS7, a synthetic *pat* gene, encoding phosphinothricine acetyltransferase and a *chi*AII gene from *Serratia marcescens* were inserted into the *Sal*I and *NotI* sites located respectively downstream of the *mas* 1' and 2' promoters. In pAR1, the bacterial signal peptide of *chi*AII was substituted by the plant leader peptide derived from potato proteinase inhibitor. A bacterial *cryIC* gene from *B.thuringinesis* sub sp.. *aizawai* 7.29 (EMBL 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, pGIF1 and pAR1 were conjugated to *Agrobacterium tumefacines* GV3101(pMP90RK), and used for transformation of alfalfa *(Medicago sativa* L. var. Regen S clone RA3) and tobacco *(Nicotaina tabacum* SR1) and potato *(Solanum tuberosum* var. Desiree) as described (D'Halluin et al., 1990; Koncz et al., 1994). To select for transformed explant, alfalfa, tobacco, and potato tissue culture media contained 40 µg /ml for alfalfa and 15 µg/ml of hygromycin for the last two were used.

Seeds of tobacco transformed according to the procedure set forth herein were deposited as patent deposits on December 23, 1996, under the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for Patent Purposes at the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland under Accession Number ATCC ____.

EXAMPLE III

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 (Fig. 2), were cloned into the *Bam*HI site of a pAEN4 vector carrying the CaMV35S gene expression cassette of pPCV91. *Arabidopsis thaliana* protoplast were isolated from root cultures and transformed by PEG-mediated DNA uptake, using 1.5 x 10^6 protoplast and 35 μg plasmid DNA in each experiment. The protoplast were harvested 48 hours after DNA uptake and lysed in SDS--sample buffer to separate proteins on 10% SDS-PAGE before immunoblotting. A polyclonal antibody used for immunoblotting was raised against a truncated CryIC δ-endotoxin carrying 756 N-terminal amino acids. Expression of CryIC in *E.coli* strains, carrying bacterial or synthetic *cryIC* genes respectively in pET-11a or 11d, was monitored-by a second alkaline phosphatase conjugated goat anti-rabbit antibody. Immunoblott analysis of proteins synthesized in plant cells was performed using an ECL kit (Amersham).

RNA (20 μg) samples isolated from leaves and petioles of alfalfa plants were separated on agarose-formaldehyde gels. *Bam*HI fragments (1.9 kb), carrying either with synthetic or bacterial CryIC sequences (Fig.2), and a *NotI* fragment with the *chiAII* gene (1.8 kb) were labeled by random-priming and used as hybridization probes. Similarly, RNA (20 μg) extracted from transgenic potato plants transformed with pAR1 was also subjected to Northern analysis, using separately *s-cryIC* as well as rDNA sequence as a specific and general probe respectively.

EXAMPLE IV

Insect bioassay

Leaf bioassay were performed with the Egyptian cotton leafworm *(Spodoptera littoralis)* and the beet armyworm *(Spodoptera exigua)* using neonate, 2-3rd, 3-4th, and 4-5-6th 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 2-3 times for each plant. The mortality of neonate larvae was scored after 3 days, whereas the mortality of larvae from 2-4th and from 4-6th 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 to 0.1% of total soluble protein as CryIC and *S. exigua* larvae of the 3-4th 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.

Potato leaves expressing about 0.02-0.05% of their total proteins as CryIC were assayed for their toxicity to *S.littoralis*. Only a single primary transformant out of 10 was less resistant to Instar III and IV larvae. This plant was later shown to contain at least 8 copies of *s-cryIC* that probably co-suppress each other.

EXAMPLE V

Expression of *cryIC* genes in *E.coli Arabidopsis*, alfalfa, tobacco, and potato

Fig. 3(A) comprises a schematic map of plant transformation vectors. The synthetic *s-cryIC* gene is cloned in an optimized gene expression cassette in pNS6 between promotor (pCaMV35S) and polyadenylation sequences

(pA35S) from the 35S RNA gene in Cauliflower Mosaic Virus. The CaMV35S promotor contains 4 repeats of the upstream enhancer region (-90 to -418) marked by open boxes. The same CaMV35S expression cassette is carried by a pAEN4, a vector used for transient expression of bacterial *b-cryIC* and synthetic *s-cryIC* genes in *Arabidopsis* protoplasts. In addition to *cryIC*, vector pNS7 contains a phosphinothricine acetyltransferase gene (pat) under the control of mannopine synthase (mas) 1'-promoter, and chitinase AII *(chiAII)* gene driven by the *mas* 2' promoter. The *chi*AII was a plant leader peptide instead of the native bacterial substitutes the original *chi*AII in pAR1, the rest of the plasmid sequence is similar to pNS7, pAR1, the rest of the plasmid sequence is similar to pNS7. pAR1 was introduced into potato and tobacco using *Agrobacterium tumefaciens* GV3101, pMP90RK. 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. Abbreviations: 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, polyadenylation signal sequences of the T-DNA encoded genes 4 and 7, respectively; pA_{ocs9} polyadenylation signal sequence of the octopine synthase gene. Open arrows label plant promoters, black boxes mark plant polyadenylation signal sequences.

Fig. 3 (B) relates to expression of *b-cryIC and s-cryIC* genes in *E. coli and Arabidopsis. Left.* The bacterial *b-cryIC* and synthetic *s-cryIC* genes were cloned respectively in vectors pET-11a and 11d, and their expression in *E. coli* was monitored with (+) or without (-) IPTG (isopropyl-β-thiogalctopyranoside) induction by

immunoblotting, 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 PEG-mediated DNA uptake with pAEN4 (1), and pAEN4- derived vectors carrying the *b-cryIC (2)* and *s-cryIC* (3) genes. Following transient expression for 48 hrs. 25 μg of soluble protein extracts prepared 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 to 756) was used as standard (2 and 20 ng.).

Fig. 3(C) relates to screening for CryIC expression in alfalfa calli, carrying the T-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 standard, in addition to control protein extracts prepared from callus tissues of wild type (wt) nontransformed alfalfa.

Fig. 3(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 (bottom lanes 1-6).

Fig. 3(E) Screening for transcripts of transgenes in leaves of soil-grown alfalfa plants carrying the T-DNA of pGIFI, 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 total RNA. The blots were hybridized respectively with *s-cryIC*, *b-cryIC* and *chiAII* probes labeled to similar specific activity. Although several GIFI 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 GIFI transformant. (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.)

Fig. 3F is a Western blot of total proteins (50 μg/lane) extracted from leaves of transgenic potato plants transformed with pAR1 and probed with anit-CryIC polyclonal antibodies. The left lane contains 10ng *E. coli* - produced CryIC (first 630 amino acids). CryIC constituted for about 0.02 to 0.05% of the total leaf proteins.

Fig. 3G is a Northern blot analysis of leaf RNA (16μg) extracted from transgenic potato plants and probed with the coding region of *s-cryIC* (shown in Fig. 3G) and then with rDNA probe to evaluate the total RNA amount loaded on each lane. Transgenic plants AR1-5, AR1-6, AR1-7, and AR1-8 revealed equal levels of *s-cryIC* transcripts while 2 fold level was found in AR1-1 and 1/1- level in AR1-2 that also revealed less production of CryIC and low resistance to the larvae.

Bacterial and synthetic *cryIC* genes were cloned respectively in vectors pET-11a and 11d, and expressed in E.coli. The synthesis of CryIC protein was monitored by immunoblotting. In comparison to cells harboring the bacterial *cryIC* gene, the expression of the synthetic gene in *E. coli* yielded a significantly lower toxin level (Fig. 3B).

The native and synthetic *cryIC* genes were inserted between promoter and polyadenylation signal sequences of the Cauliflower Mosaic Virus (CaMV) 35S RNA gene in the plant gene vector pAEN4. In pAEN4 the 5'-ends of *cryIC* genes were fused to untranslated Ω leader sequences of the Tobacco Mosaic Virus (TMV) to enhance the translation of mRNAs, whereas the upstream CaMV 35S promoter was supplemented with 4 repeats of the enhancer domain (-90 to -418), to stimulate the transcription of chimeric

genes in plants (18). The *cryIC* genes were introduced by PEG-meditated transformation into *Arabidopsis* protoplasts, and the accumulation of CryIC toxin was monitored by immunoblotting following transient gene expression (Fig. 3B). In protoplasts carrying the bacterial gene no toxin was detectable, whereas cells transformed with the synthetic gene accumulated significant amount of CryIC protein (Fig. 3B).

The *cryIC* genes were transferred into pPCV91, a T-DNA-based transformation vector carrying a selectable hygromycin resistance (hpt) gene. In the dual gene expression cassette of pPCV91 (Fig. 3A), a synthetic phosphinothricine acetyltransferase (*pat*) gene 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* was inserted downstream of the *mas* 2' promoter, because our previous studies indicated that chitinases may enhance the insecticidal activity of *Bt* toxins by destroying the chitinous peritrophic membrane of insect midgut. The pPCV91 constructs, carrying the native, or synthetic, *cryIC* genes either alone, or in combination with a pat *and chiAII* genes, were introduced by *Agrobacterium*-mediated transformation into alfalfa, tobacco, and potato. From tobacco and potato 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. 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. 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.

EXAMPLE VI

Assaying Resistance of *cryIC* Transgenic Plants 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 leaf worm (*S. littoralis*). 15 out of 27 NS6 transformants, and 14 out of 32 NS7 transformants produced 100% mortality of larvae (Fig. 4*A*, Table 2). 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 armyworm *(S. exigua)* also caused 100% mortality of larvae throughout their development (Fig. 4*C-D)*, Table 2). Screening of the NS7 transgenic alfalfa demonstrated that 15 out of 32 tested plants (47%) exhibited the high level 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 mg 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.

About 80 hygromycin resistant potato plants (cultivars Desiree) were regenerated and 9 of them were subjected to molecular analysis and bioassays. Two out of 9 plants did not express CryIC and were sensitive to *S.littoratis* larvae. The rest of the 7 plants displayed

resistance to all instar larvae. While the plant AR1-2 was less resistant, the other 6 plants were totally resistant to all instar larvae (Fig. 5) and contain 1-3 inserted copies of *s-cryIC*. At least eight copies of *s-cryIC* were detected in the plant AR1-2. The potato-produced CryIC was less susceptible to proteolysis by the *S. littoralis* gut proteases (Fig. 5), but denaturation and renaturation *in vitro* render it susceptible to proteolysis.

From 63 NS7 tobacco transformants 42 lines (66.6%) were resistant to 1.0% BASTA, Proper Mendelian segregation of a BASTA and hygromycin resistance markers was confirmed after selfing 11 transgenic tobacco lines. From these BASTA resistant plants 10 stocks were assayed by immunoblotting and found to produce 0.1-0.2% of leaf soluble proteins as CryIC toxin (Fig. 3*D*) and resulted in 100% mortality of *S. exigua* larvae. 3 from these lines were used in bioassays with *S. exigua* and found to cause 100 % mortality of larvae from different developmental stage.

The insecticidal assays showed no difference between plants carrying the cryIC gene alone or in combination with the *chiAII* gene. A synergistic effect between chitinase AII and CryIC toxin could have escaped our detection because toxin levels as low as 0.01% of total plants were sufficient to kill all larvae. To imitate field conditions, CryIC expressing plants were infested by 15-20 larvae of 3rd-4th instar stage in 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. Infestation of a mixed population of wild-type and transgenic plants carrying the synthetic cryIC gene resulted in devastation of wild-type, but yielded no apparent colonization of worms on the CryIC toxin expressing plants in the population.

Similar results were obtained by infesting detached leaves from these plants with larvae of *S. exigua*.

EXAMPLE VII

Screening for Spodoptera Resistance of Transgenic Plants

Fig. 4(A) shows an insecticidal assay with neonate larvae of *S. littoralis* reared for 2 days on leaves from non-transformed alfalfa (*M. saliva*, top) and NS7 transgenic (bottom) plants.

Fig. 4(B) shows "free choice" bioassays with leaves from wild-type and transgenic alfalfa plants. In the plate to the left 10 larvae of *S. exigua* (3rd 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.

Fig. 4(C-D) shows leaves from tobacco (*C*) and alfalfa (*D*) plants were used for feeding of five fifth instar larvae of *S. exigua* for 10 hrs. Petri dishes to the left in (*C-D*) contained leaves from nontransformed plants. Leaves shown in Petri dishes to the right in (*C-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.

Fig. 4(E) shows transgenic NS7 (left, Fig. 3*D*, lane 2) and nontransformed alfalfa (right) plants were infested with 15 larvae of *S. exigua* (3-4th instar stage) for 6 days.

Fig. 5 demonstrates the resistance of transgenic potato leaves resulted from pAR1 introuction to 2nd, 3rd and 4th instar larvae of *S.littoralis*. Leaves of plant

AR1-2 less resistant while the control leaves were partially or totally consumed by the young and older larvae respectively. The leaves were photographed after 24h of exposure to the larvae.

Fig. 6 is a Southern analysis of transgenic potato plants showing the integration of 1 to at least 8 copies of CryIC. The plant number is indicated above the lanes, probes-below the panels.

Fig. 7 is a Western blot performed with total proteins extracted from transgenic potato leaves or from *E.coli* expressing the 630aa CryIC, and incubated for 2, 10, 20 min. with gut juice of the 4th instar larvae of *S.littoralis*. The *E.coli* produced CryIC is more susceptible to proteolysis. Denaturation of the plant CryIC analysis its resistance to proteolysis.

The control of *Spodoptera* (armyworms) by transgenic alfalfa plants shown in Table 2.

TABLE 2

	Mortality of *S. littoralis* neonate larvae		
	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%)

Mortality of *S. Exigua* larvae fed on leaves of plants with 0.02-0.1% CryIC toxin level			
Transgenics	**NS6**	**NS7**	**Time of scoring**
	(number of plants tested)		**(days)**
Instar 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

Out of 60 NS7 transgenic alfalfa plants 14 lines were found to be resistant to 0.1-0.2% BASTA. From these plants 9 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 in %.

Although only preferred embodiments are specifically described herein, it will be appreciated that many modifications and variations of the present invention are possible in light of the above teachings and within the purview of the appended claims without departing from the spirit and intended scope of the invention.

WHAT IS CLAIMED IS:

1. A gene encoding a *Bacillus thuringiensis* toxin protein said gene having the nucleotide sequence illustrated in FIG. 2 (SEQ.ID.NO:1)

2. A plant cell, the genome of which comprises a synthetic gene that encodes an N-terminal fragment of between about 615-630 amino acids, said fragment derived from DNA encoding a *Bacillus thuringiensis* insecticidal crystal protein of about 135 kD which has been truncated.

3. The plant cell of claim 2 wherein said insecticidal crystal protein is a CryIC protein.

4. The plant cell of claim 2 wherein said synthetic gene is the gene of claim 1.

5. Tobacco seeds comprising plant cells of claim 2 deposited with the ATCC having accession number _____.

6. A water soluble insecticide comprising a protein having the amino acid sequence illustrated in Fig. 2 (SEQ. ID. NO:2).

7. An insecticide comprising a protein expressed by a plant transformed by the gene of claim 1.

8. A dicotyledonous plant genetically transformed by the gene of claim 1.

9. The plant according to claim 8, wherein said plant is selected from the group consisting of alfalfa, tobacco, and potato.

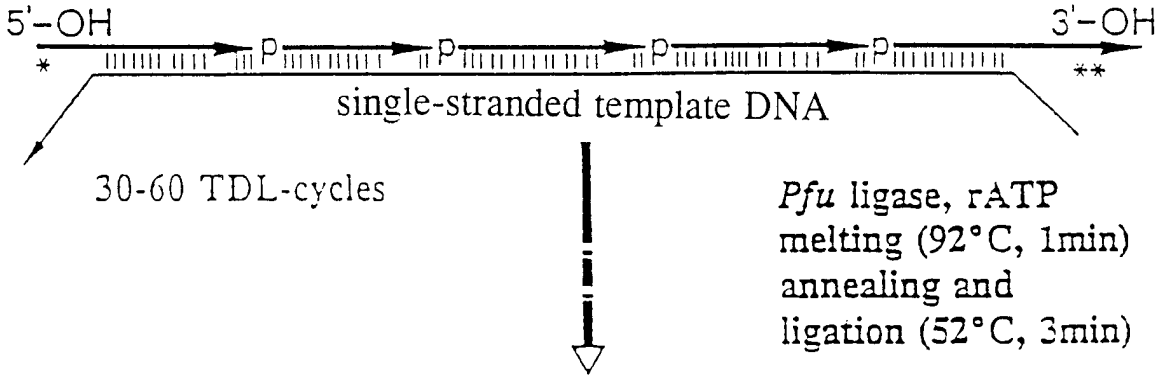
10. A transgenic, dicotyledonous plant which expresses a water soluble insecticidal crystal protein.

11. The plant according to claim 10 wherein said protein is a CryIC protein.

12. The plant according to claim 11 wherein said protein comprises the amino acid sequence illustrated in Fig. 2 (SEQ. ID. No:2).

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

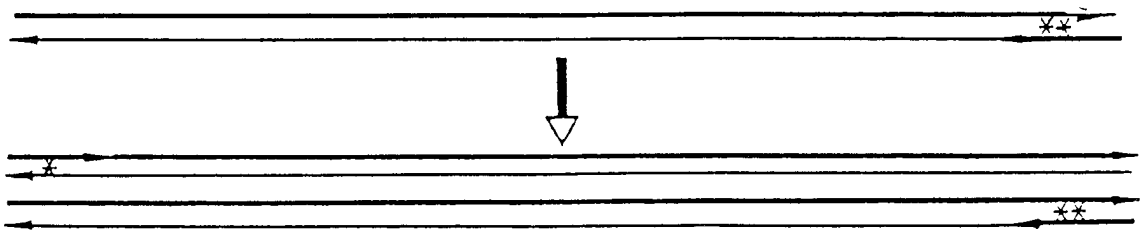


Fig. 1

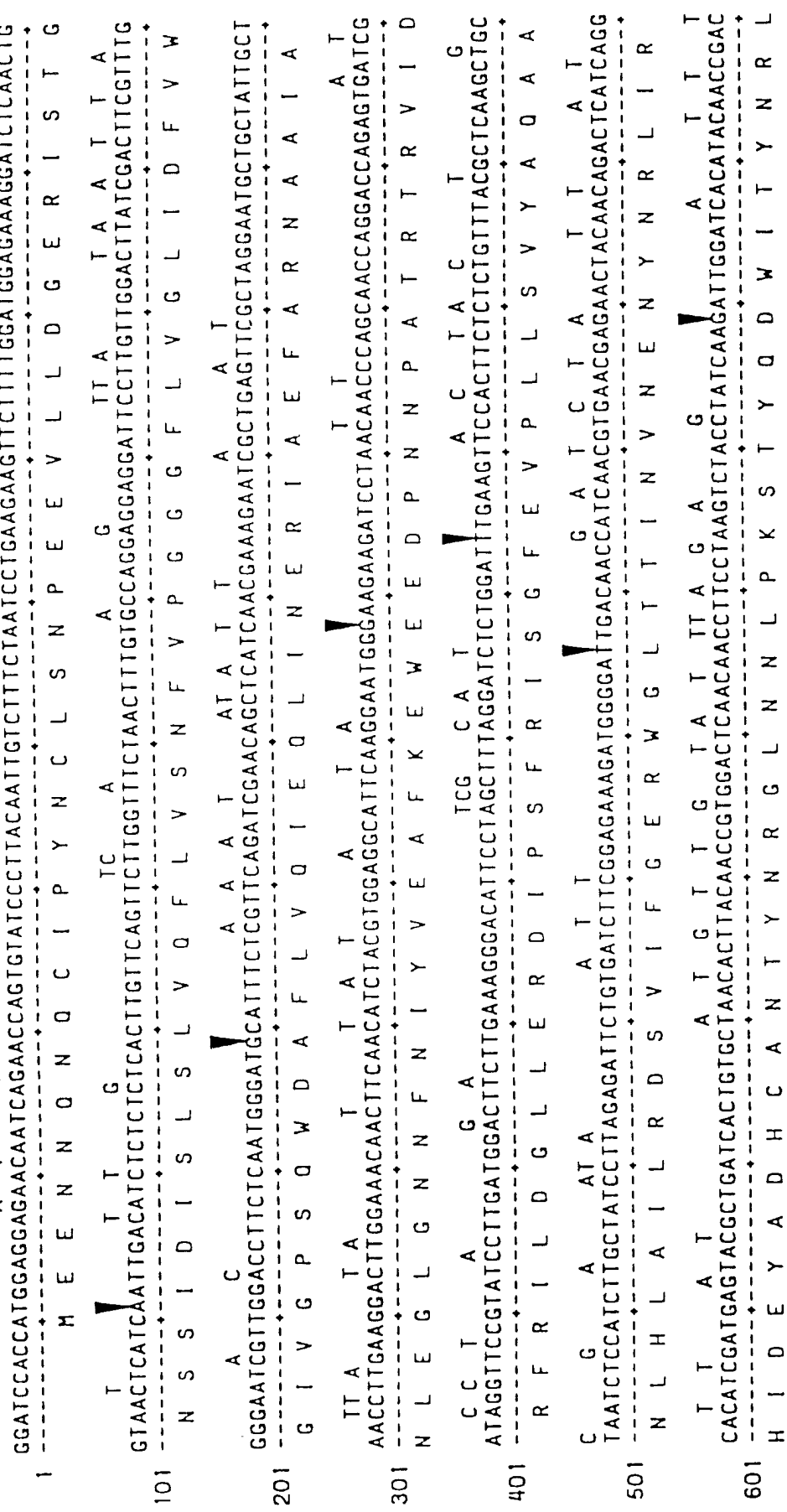


Fig. 2

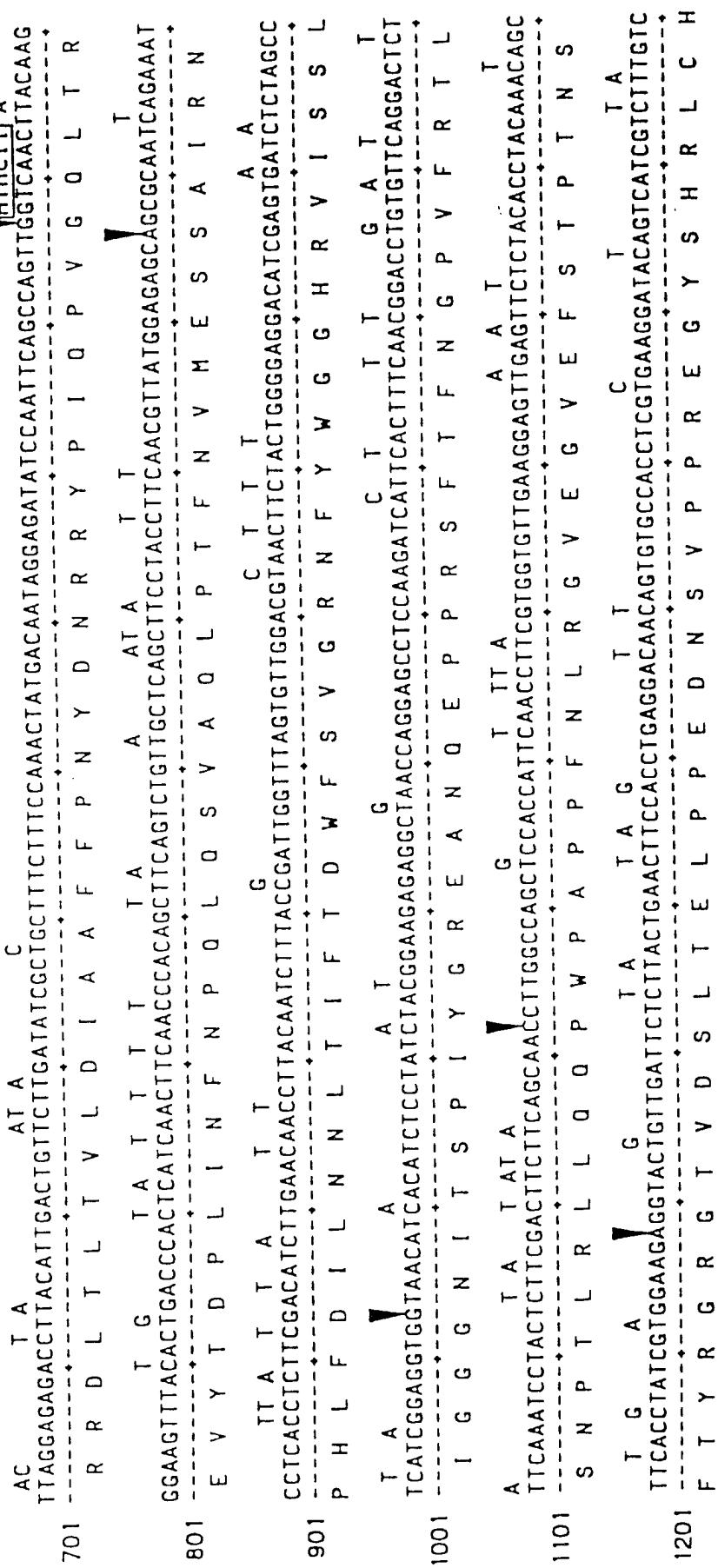


Fig. 2 (cont'd)

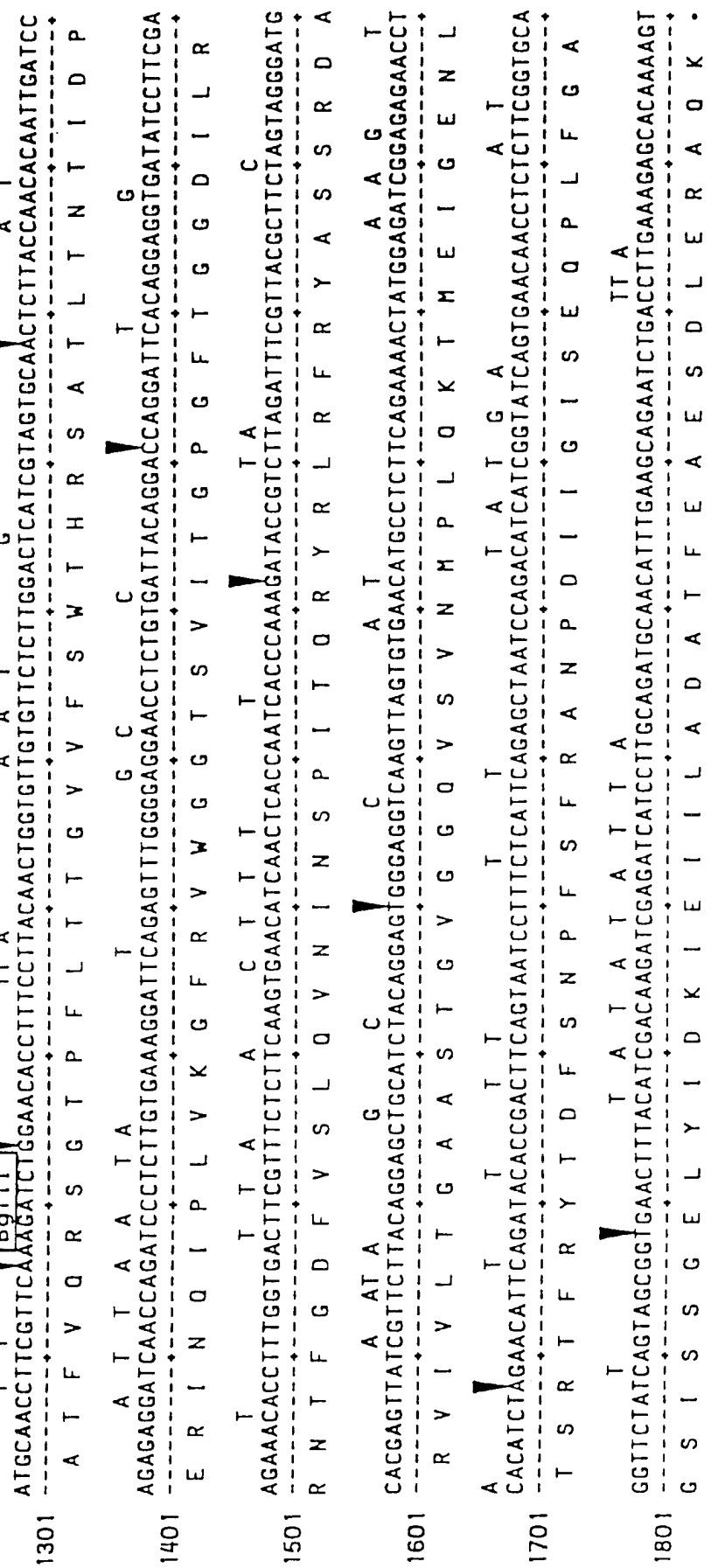
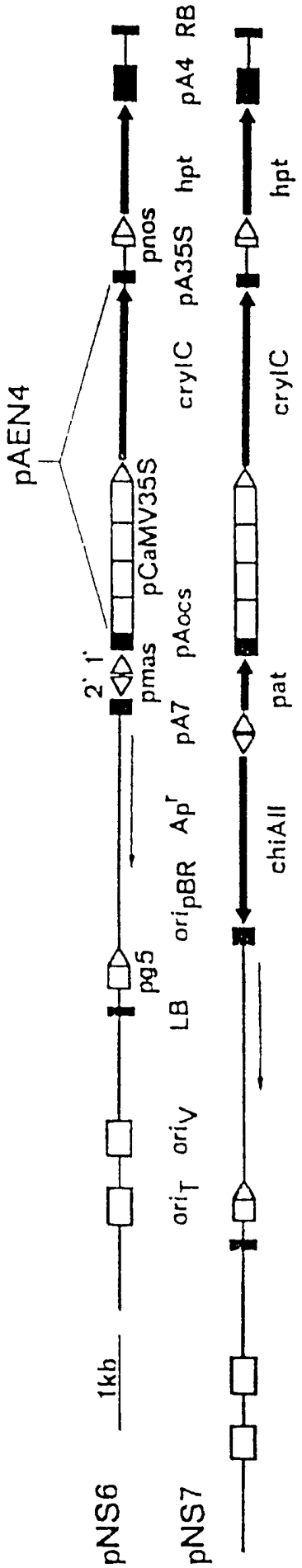


Fig. 2 (cont'd)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2' 1'
pmas
pCaMV35S
pAocs
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pnos
cryIC
pA35S
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pA4
RB
pg5
LB
ori pBR
Ap r
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cryIC
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ori T
ori V
1kb
pNS6
pNS7

Fig. 3 A

Fig. 3 B

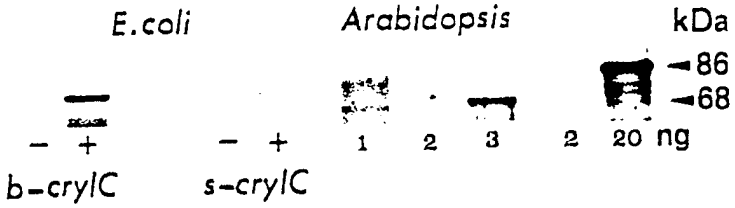


Fig. 3 C

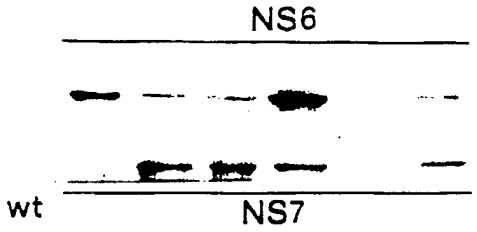
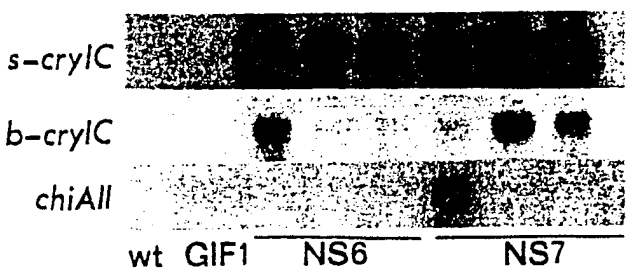
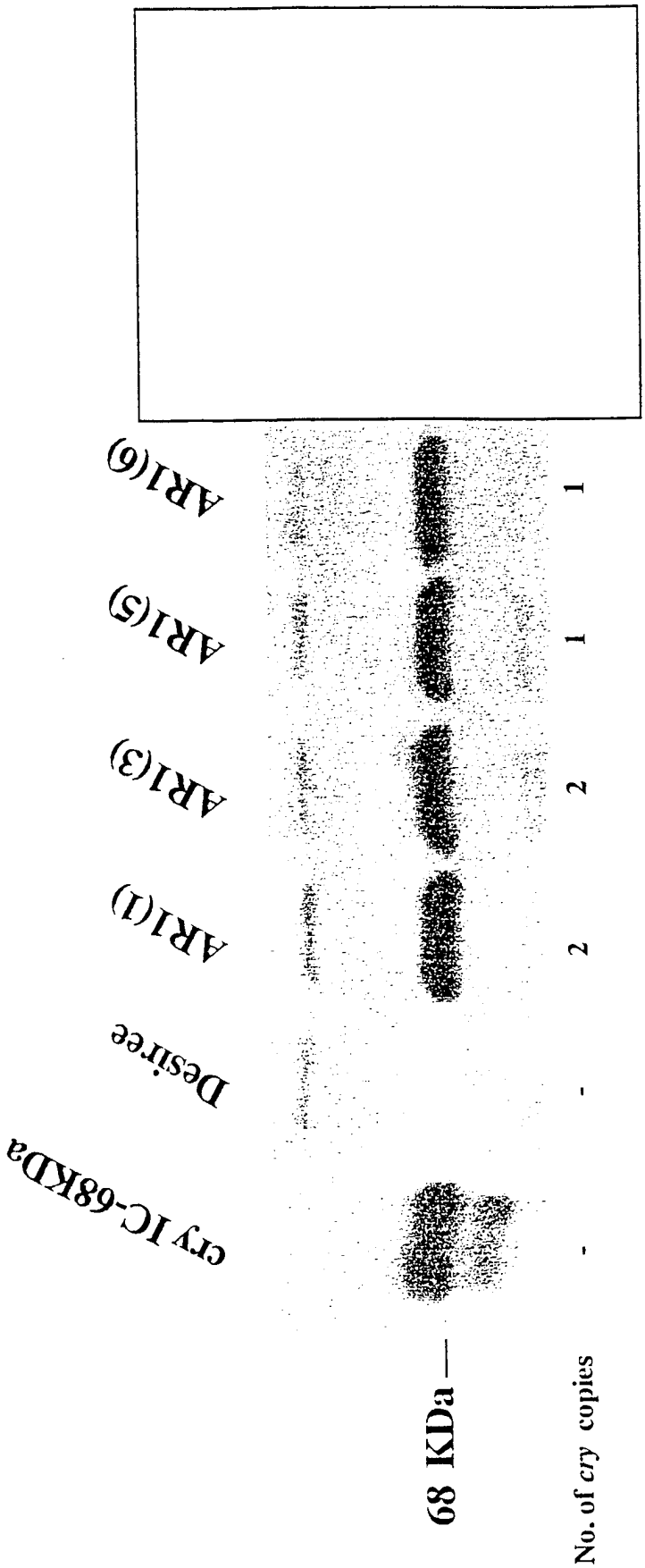


Fig. 3 D

Fig. 3 E




cry1C-68KDa
Desiree
ARI(1)
ARI(3)
ARI(5)
ARI(6)
68 KDa
No. of cry copies
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-
2
2
1
1

Fig. 3 F

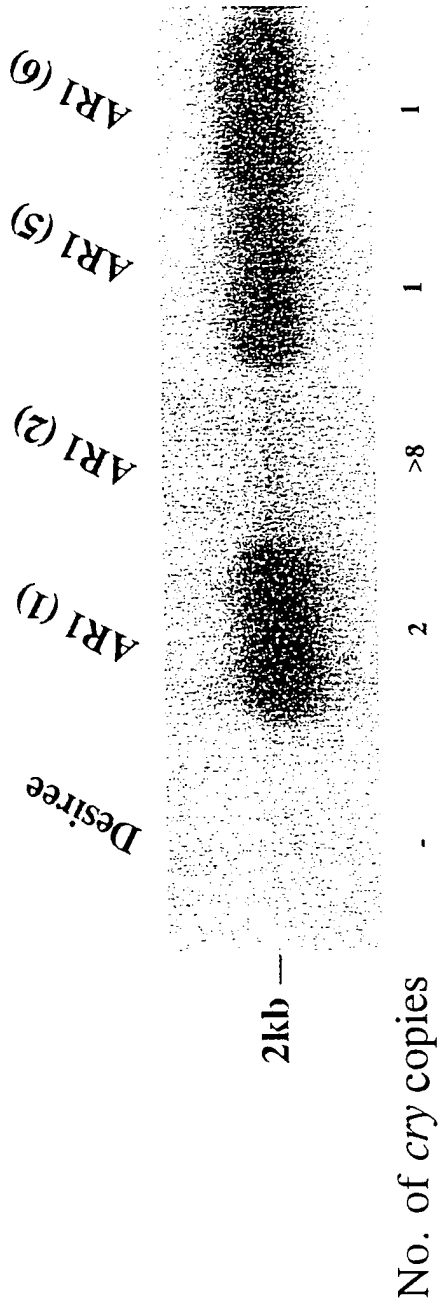


Fig. 3 G

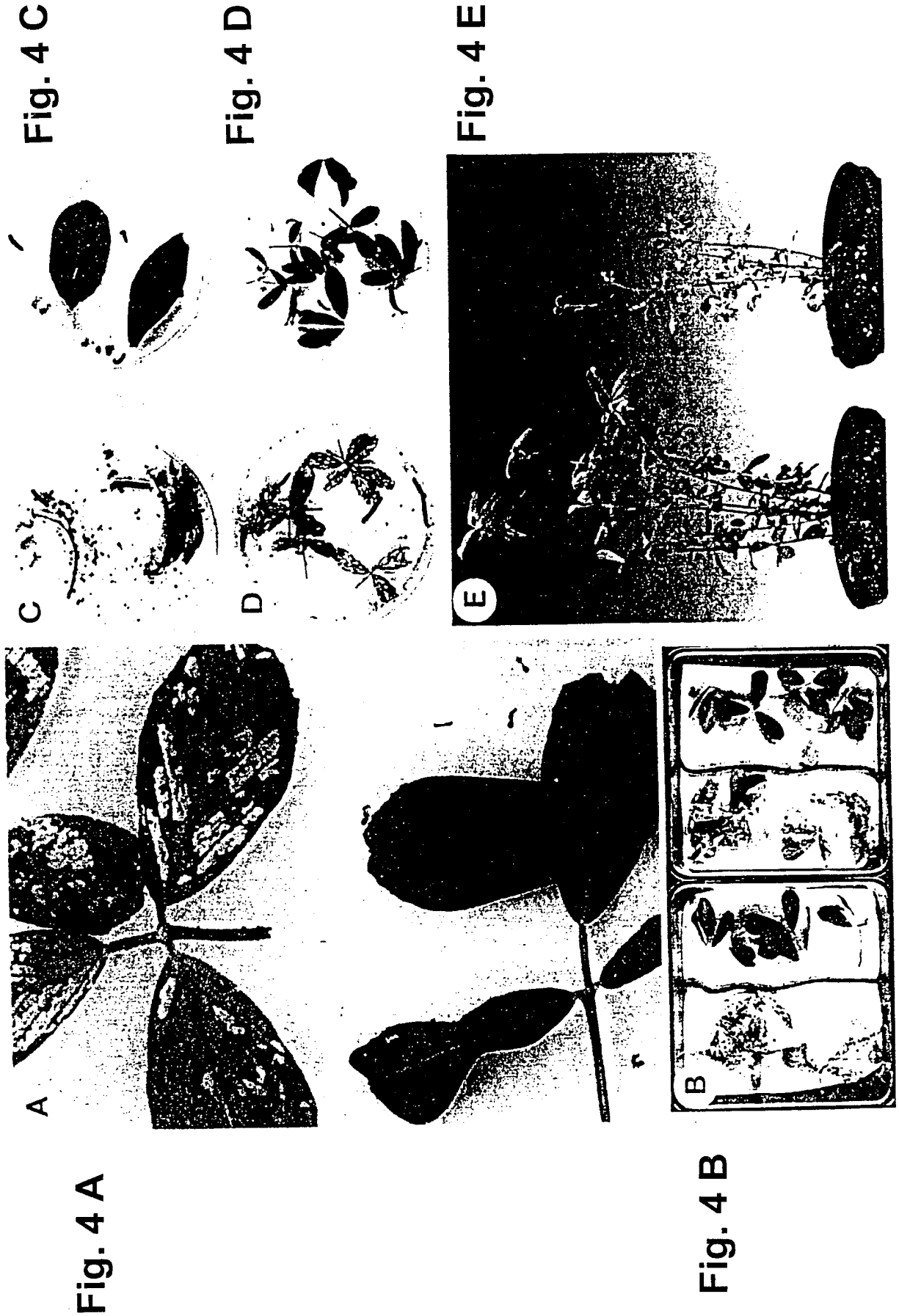


Fig. 4 C
Fig. 4 D
Fig. 4 E
C
D
E
A
B
Fig. 4 A
Fig. 4 B

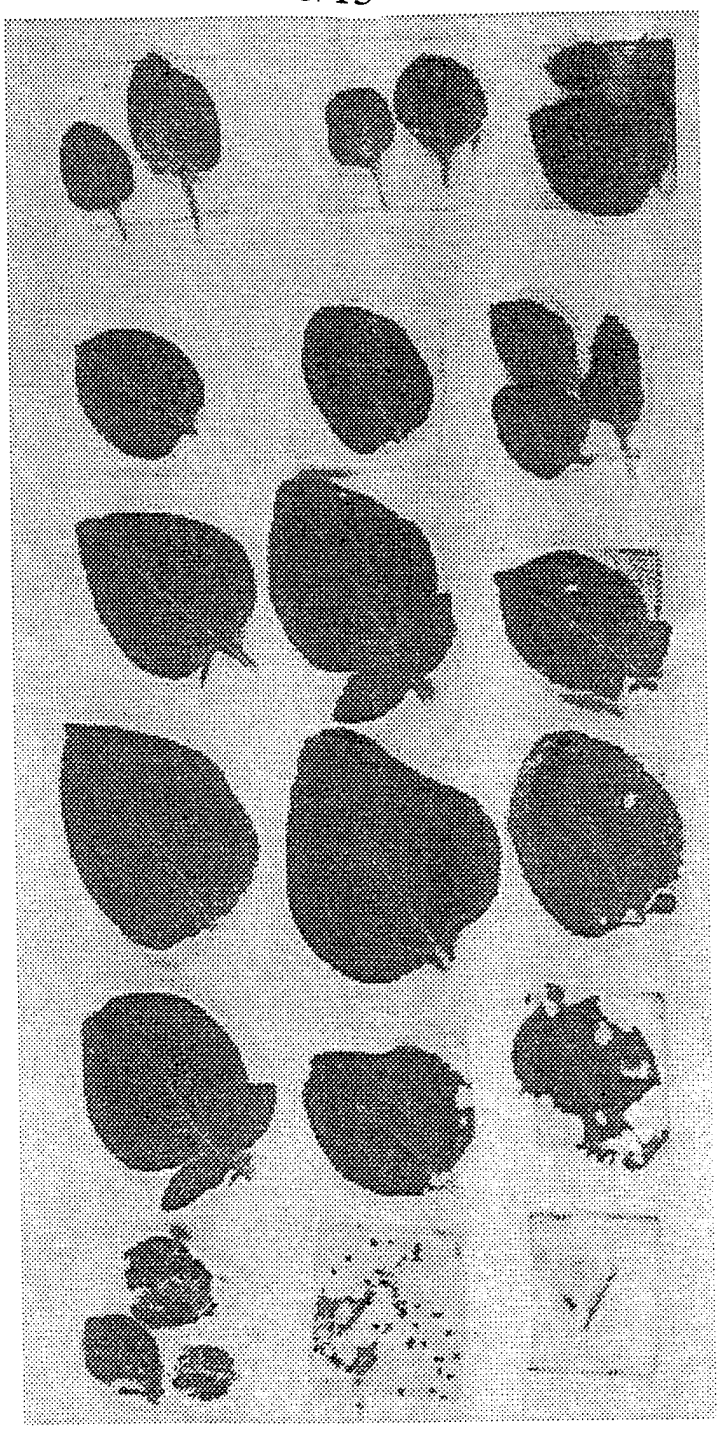

Control (Desiree)
Transgenic plants
1
2
3
4
5
Instar II
Instar III
Instar IV
No. of cry copies
-
8
2
2
1
1

Fig. 5

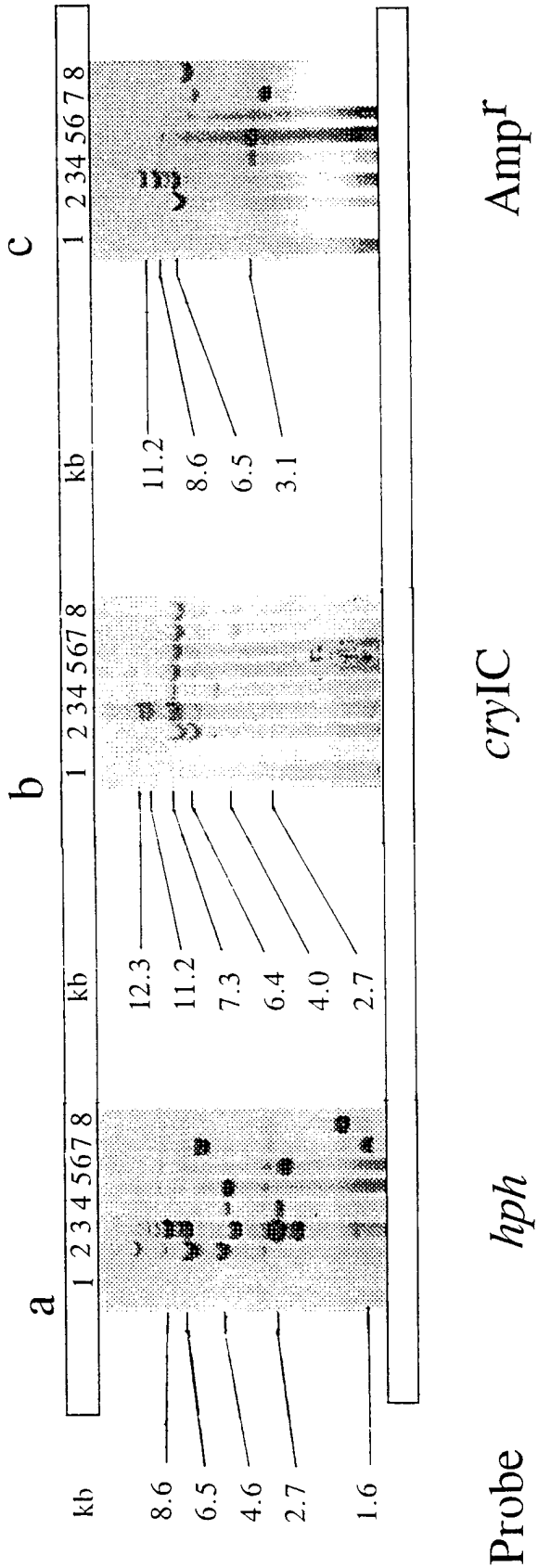
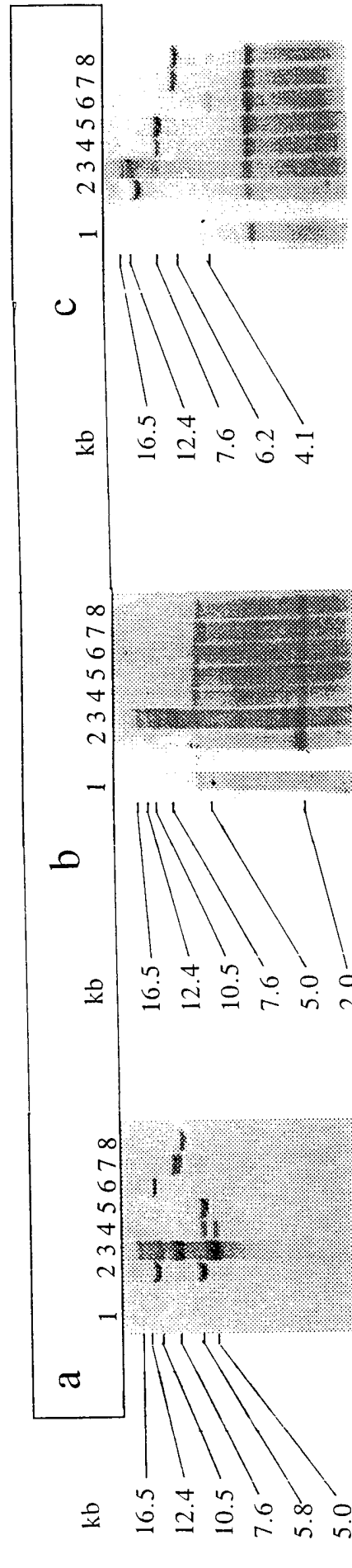

a
b
c
1 2 3 4 5 6 7 8
kb
12.3
11.2
7.3
6.4
4.0
2.7
11.2
8.6
6.5
3.1
8.6
6.5
4.6
2.7
1.6
Probe
hph
cryIC
Ampr

Fig. 6 A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12.4
10.5
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5.0
Probe
hph
b
1 2 3 4 5 6 7 8
kb
16.5
12.4
10.5
7.6
5.0
2.0
cryIC
c
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kb
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Ampr

Fig. 6 B

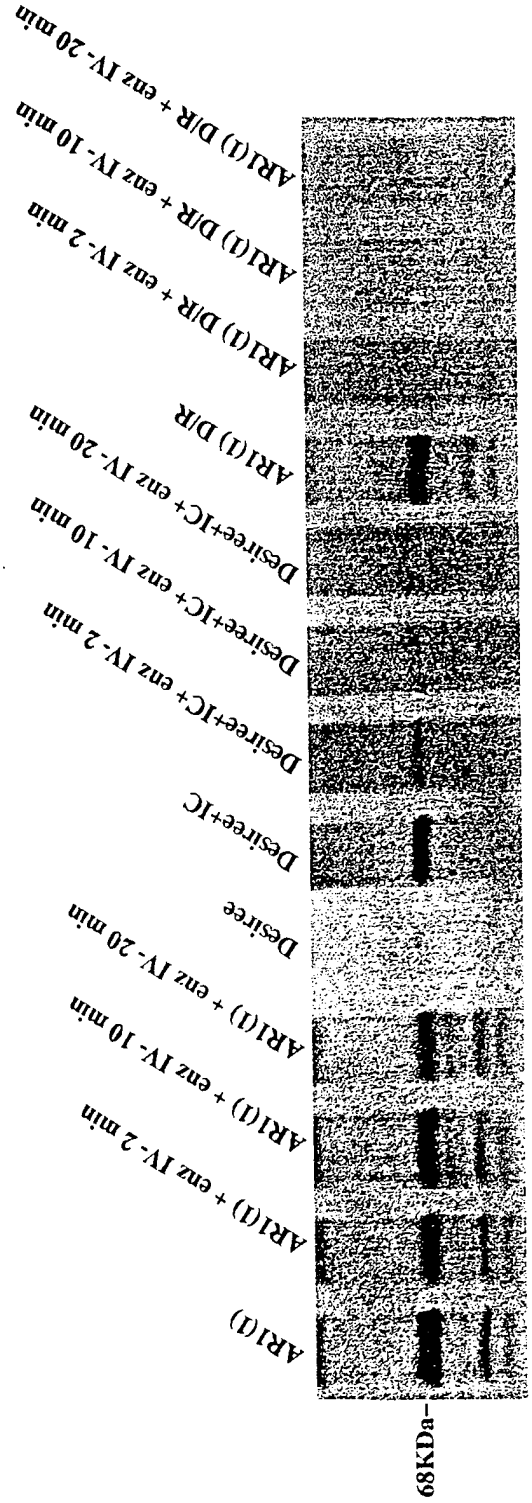
ARI(I)
ARI(I) + enz IV- 2 min
ARI(I) + enz IV- 10 min
ARI(I) + enz IV- 20 min
Desiree
Desiree+IC
Desiree+IC+ enz IV- 2 min
Desiree+IC+ enz IV- 10 min
Desiree+IC+ enz IV- 20 min
ARI(I) D/R
ARI(I) D/R + enz IV- 2 min
ARI(I) D/R + enz IV- 10 min
ARI(I) D/R + enz IV- 20 min
68KDa

Fig. 7