Transgenic crop plants expressing synthetic *cry9Aa* gene are protected against insect damage

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Abstract

A synthetic gene sequence of *cry9Aa* was made to achieve high expression levels in a plant cell. Tobacco, potato, cauliflower and turnip rape plants were transformed with this synthetic gene driven by the double 35S promoter using *Agrobacterium tumefaciens* LBA4404. The presence and expression of the synthetic *cry9Aa* gene was evaluated in Southern, Northern and Western analysis and with insect bioassays. The expression of the gene in tobacco plants reached a level of 5 pg of mRNA per 1 mg of total RNA and 0.3% of soluble protein or 1.4 mg of Cry9Aa protein per 1 g of leaf material. The expression level in the other species was three to ten times lower. Tobacco plants were also transformed with a truncated native *cry9Aa* gene construct and with a translational fusion construct of the truncated native *cry9Aa* and the *uidA* (GUS) gene sequence. The constructs were transformed in tobacco plants under the control of the same promoter as the synthetic *cry9Aa*. The expression level of the native *cry9Aa* gene constructs ranged from 0.03 to 1 pg of *cry9Aa* mRNA per 1 mg of total RNA. The protein was undetectable in Western analysis. In comparison to the native constructs the expression level of the synthetic *cry9Aa* gene was five to ten times higher at the mRNA level and at least 50 times higher at the translational level. Bioassays against *Plutella xylostella* performed with transgenic cauliflower showed high insecticidal activity of the plants expressing the synthetic *cry9Aa* gene. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

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1. Introduction

Bt delta-endotoxins encoded by *cry* genes of *Bacillus thuringiensis* are known as the most effective proteinaceous insecticides used in agriculture. These toxins are widely used as bacterial preparations, which are sprayed on field plots to control varied pest insects [1]. However, these preparations are expensive and degrade fast under field conditions. To overcome these problems *cry* genes have been transformed into different plant species to develop insecticidal crops. First attempts to transform native *cry* sequences have failed mostly because AT-rich nucleotide sequences of the genes are not desirable for expression in plants. In fact, full size native *cry* genes encoding protoxins did not express in plants at all [2]. Therefore, the native *cry* genes were truncated to encode only the active trypsin-stable core of the Bt toxins. However, the truncated genes still had many mRNA processing signal-like sequences and bacillar codon preference, which effectively reduced their expression in plants [2–4], and thus native *cry* gene sequences transformed into plant nucleus did not provide high enough expression to control certain insect pest species in field trials [5,6].

Partial, site directed modifications in the gene sequence enhanced the expression by several fold but still the expression was not enough for protection against certain pest insect species [7,8]. Only
full resynthesis of a truncated cry gene can provide high-level expression of the gene introduced in the genome of transgenic plants. Synthetic cry genes have been transformed to several crops that are used in agricultural production. The first synthetic genes were cry1Ab and cry1Ac [7,9] and cry3Aa [10]. The expression of the synthetic cry gene constructs was reported to be about 0.1–0.3% of soluble protein corresponding to about 1 μg of toxin protein per 1 g of fresh leaf material.

The main concern of using insecticidal transgenic plants in insect control is the risk of development of resistance to the Bt toxin by insect pests. To overcome this problem pyramidal expression of Cry toxins with different receptor binding mechanisms have been used [11]. Up to today, the limitation to the latter strategy is that there are not many different cry genes available or characterized that would be useful in resistance management studies.

Here we report the synthesis of cry9Aa gene, its expression level and the insecticidal effect of its protein product in transgenic plants. The toxin was found as a crystal protein of B. thuringiensis spp. galleria str. 11–67 [12] and subsequently the cry9Aa1 (cryIG gene in the old nomenclature) gene having specificity to the larvae of Galleria melonella was cloned [13,14]. The Cry9Aa1 toxin showed an insecticidal spectrum different from Cry1Ac and Cry1Ca toxins in earlier performed bioassays (Kuvshinov et al., not published). The protein was found toxic to several insect species: Pieris brassica, Plutella xylostella, Manduca Sexta (not published) and Phthorimaea operculella [15].

The present article describes the modification, synthesis and expression of a synthetic cry9Aa gene in plants.

2. Materials and methods

2.1. Bacteria, plants and insects

Escherichia coli strain XL1 was used for the cloning of the DNA constructs. Agrobacterium tumefaciens LBA4404 [16] was used in plant transformation. The transformed species included: tobacco Nicotiana tabaccum cv. Samsung, potato Solanum tuberosum cv. Pito, cauliflower Brassica oleracea var. botrytis cv. Asterix and turnip rape Brassica rapa var. oleifera cv. Valtti. Bioassays were performed against diamondback moth, P. xylostella. Anthony Shelton from Cornell University, USA, kindly provided lines of insects resistant against Cry1Ac and Cry1Ca toxins for bioassays.

2.2. DNA synthesis and cloning

DNA synthesis and truncation of the gene were performed by high fidelity PCR using Pfu polymerase. Oligonucleotides used in PCR were 55–82 nucleotide long and were ordered from DNAgency and Operon Technologies, Inc., USA. The DNA sequences were cloned in pUC19, pBluescript and pUK21 vectors. The gene constructs were cloned in the plant transformation vector pGPTV-HPT [17].

2.3. Plant transformation

Plants were transformed with A. tumefaciens strain LBA4404 carrying binary pGPTV-HPT pBIN19-based vector [18,19]. Tobacco plants were transformed by leaf disc inoculation [20], without using nurse culture and with 30 mg/l hygromycin selection instead of kanamycin. Potato plants were transformed by stem segment inoculation [21] and cauliflower by hypocotyl segment inoculation [22]. Turnip rape plants were transformed by inoculating stem segments of greenhouse grown plants [23]. About 20 antibiotic resistance tobacco lines for each construction, and about 10–15 transgenic lines of other plants were taken in consecutive analysis.

2.4. Molecular analysis of gene expression

Greenhouse grown transgenic plants were tested by molecular analysis for cry9Aa gene expression. Total DNA, RNA and protein isolation was performed from tissues of mature leaves. Southern blot analysis was performed using plant DNA isolated with Quiagen DNeasy Plant kit. DNA samples (3–5 μg) were loaded in agarose gel, run and blotted in vacuum blotter on Boehringer Mannheim positively charged nylon membrane. The membrane was hybridized and developed according to the supplier’s instructions (Boehringer Mannheim, ‘The DIG user’s guide for filter hybridization’). DNA probes for hybridization were amplified in PCR with Digoxigenin-dUTP.
Northern blot analysis was also performed according to the supplier’s recommendations (Boehringer Mannheim, ‘The DIG user’s guide for filter hybridization’). Total RNA preparations were isolated using the Quiagen RNeasy kit. Three micrograms of total RNA was run in an agarose gel and blotted on the positively-charged nylon membrane. The membrane was hybridized and developed according to supplier’s instructions (Boehringer Mannheim, ‘The DIG user’s guide for filter hybridization’). The RNA probe was synthesized with T7 RNA polymerase using Digoxigenin-UTP. pBluescript vector containing cry9Aa gene sequence served as the template. The probe was synthesized in opposite direction to the gene sequence (minus strand). Control mRNA molecules were synthesized with T3 RNA polymerase from the same template vector in the direction of the gene sequence (plus strand). Native truncated and synthetic gene sequences in the pBluescript II SK (Stratagene) vector were digested with different restriction enzymes to produce different size control mRNAs. Different quantities of the control mRNAs were mixed with 3 μg of total RNA of non-transformed plants and loaded in the gel to compare the signals of transgenic plant lanes with the controls and to evaluate the level of the cry9Aa mRNA expression.

Western blot hybridization was performed using rabbit polyclonal antibodies against crystal proteins of B. thuringiensis spp. galleriae str. 11–67. The antibodies were preabsorbed with plant proteins. The proteins for preabsorption were extracted from non-transformed plants as acetone-precipitated powder from the solubilized protein fraction in extraction buffer as described below. The protein powder was resuspended in antibody serum at 1% (w/v) concentration and incubated in room temperature for 15 min.

Protein samples were produced as follows. One gram of leaf material was ground in liquid nitrogen and resuspended in 2 ml of protein extraction buffer (50 mM Tris (base), NaOH (titrated up to pH 12.5), 0.4 M Urea, 0.1 M Thiourea, 2 mM DTT, 0.5% Tween-20, 0.5% Triton-X100, and 4% Mercaptoethanol). The mixture was warmed up to 60°C for 5 min, refrozen in liquid nitrogen and thawed. This procedure was repeated twice. Debris was removed by centrifugation and the supernatant was precipitated with four volumes of cold acetone — 20°C. The protein precipitate was washed two times with cold acetone and, finally, dried in vacuum. The protein powder was weighed, resuspended and dissolved in loading buffer (50 mM Tris—HCl pH 8.5, 2% SDS, 0.2% Bromphenol blue, 20% Glycerol, 4% Mercaptoethanol) at a concentration of 1 μg of the precipitate in 40 μl of buffer. The suspension was boiled in water bath for 10 min. The insoluble fraction was removed by centrifugation and the supernatant was used for further analysis. Concentration of the total protein in the sample from supernatant was measured by Bradford assay. The samples were loaded and run in 0.75–1.5 mm thick 8% denaturing SDS-polyacrylamide gel. After running the gel was blotted on nitrocellulose membrane in BioRad semi-dry bloter. The membrane was then blocked in 0.5% BSA and hybridized with the antiserum. The signal was developed using alkaline phosphatase antirabbit conjugate. Cry9Aa protein expressed from the truncated toxin gene in E. coli was solubilized and the crude protein extract served as a control. Concentration of the protein expressed in E. coli was measured in Coomassie stained polyacrylamide gel comparing the band intensity with a BSA concentration series. The Cry9Aa protein was mixed with non-transformed plant protein and used in different concentrations to estimate the expression level of the gene in plants.

2.5. Bioassays

Transgenic plants of cauliflower and turnip rape were used in bioassays against larvae of wild type and Cry1Ac or Cry1Ca toxin resistant lines of P. xylostella [24]. Leaf sheets were fed to second or third instars and insect mortality was recorded. Five larvae were placed on a leaf sheet, which was changed to a fresh one every 2 days. The leaf sheets with the larvae were kept at room temperature in 50-ml plastic tubes covered by miracloth and placed on the top of the opening of a 100-ml jar half-filled by water. Each test was repeated two to four times. In another test the transgenic and control cauliflower plants were placed in a chamber with P. xylostella larvae. The insecticidal effect of the plants was registered as the number of lesions on the leaves after 7 days.
3. Results

3.1. Modifications to and synthesis of the cry9Aa gene

The synthetic DNA sequence was designed based on the amino acid sequence of the active N-terminal part of Cry9Aa1 parasporal toxin of *B. thuringiensis* spp. *galleriae* [14,15] determined by the trypsin processing sites. Modification was performed to improve the expression level in higher plants. First the codon preference was changed according to the compiled codon usage tables for *Brassica* species, dicots, monocots and higher plants (the tables not shown). The codon tables for *Brassica* plants were compiled using computer software (Wisconsin Package Version 10.1, Genetics Computer Group (GCG), Madison, WI). Dicot and monocot plant codon usage tables were compiled from available tables [25]. The sequence of codons was constructed manually with a preference to dicots and *Brassica* species. Codons to monocots [26] were also avoided. Preferred start codon context of higher plants was also introduced in the sequence. In all, the nucleotide sequence was changed by 24%.

The newly constructed sequence was revised to remove RNA processing-like signal sites. The sequences searched for included putative polyadenylation, splicing and mRNA destabilizing signals [27–29]. Undesirable sequences were replaced by translationally neutral nucleotide substitutions. The gene sequence was also checked for the presence of repetitions and palindromes in order to avoid undesirable secondary structure formation. Finally the sequence was divided into five 360–430 base long fragments with introduction of restriction sites without changing the amino acid sequence. The final synthetic sequence is shown in Fig. 1.

Each fragment was synthesized in high fidelity PCR using Pfu polymerase. The synthesis of each fragment was performed in three to four consecutive expanding PCRs in the following order: first — extension by central pair of primers, then — second more distal pair and finally terminal pair of primers. The first and second PCR products were used as templates for the following pair of primers. The orientation and order of the oligonucleotides is shown with arrows in Fig. 1 for the first synthesized fragment as an example. The final PCR product of the fragment was cloned in an appropriate cloning vector and sequenced. The synthesized fragments of the gene were consecutively ligated into the entire gene sequence.

The native *cry9Aa* sequence was truncated by high fidelity PCR. The primers used were designed to match the 5’ and 3’ ends of nucleotide sequence encoding for the amino acid sequence of trypsin-stable core domain of the toxin. Restriction sites convenient for cloning were introduced into the primers. The PCR product was cloned in the same plant transformation vectors as the synthetic gene. The truncated gene sequence was also fused in the same translational frame with the *uidA* (GUS) gene. The fusion construct was cloned the same way as the other constructs.

3.2. Insertion of cry9Aa gene in cloning and plant transformation vectors

The synthetic *cry9Aa* gene sequence was cloned in pBluescript SK (Stratagene) between the BamHI and *XhoI* restriction sites in a translational frame of LacZ α-peptide. The protein product was expressed in *E. coli* from this construct and identified in Western analysis. The solubilized total protein of *E. coli* containing the expressed Cry9Aa toxin was used as a positive control in Western analysis of the transgenic plants.

The synthetic as well as the native truncated and GUS-fused sequences of the *cry9Aa* gene were cloned in the plant transformation vectors (Fig. 2). The genes were placed under the control of double 35S promoter (35S:Sp) [30] linked with the AMV-leader from alfalfa mosaic virus [31]. The vectors carried the *hpt*-hygromycin phosphate transferase — gene, which confers resistance to hygromycin. The vectors were transformed into *A. tumefaciens* LBA4404, which was used in plant transformation.

3.3. Molecular analysis of cry9Aa gene expression in plants

Transgenic plants were tested in Southern analysis for the presence of transgene insertion, as well as in Northern and Western analysis for the expression of mRNA and protein product of the *cry9Aa* gene.
3.3.1. Tobacco plants

Tobacco plants were transformed with synthetic, native truncated and GUS fused sequences of cry9Aa gene. The Southern blot analysis confirmed transgene insertion in the genome. The analysis of the plants showed that at least 1/3 of the plants contained one transgene insertion in their genome (data not shown). Plants positive in Southern analysis were tested on a Northern blot for the presence of cry9Aa mRNA.

The three transgenic plants carrying the synthetic cry9Aa gene showed an average expression of about 5 pg of cry9Aa mRNA per 1 µg of total RNA (Fig. 3A). The western blot showed that average expression of Cry9Aa protein in these lines was about 0.2% of total soluble protein or 1 µg per 1 g of leaf material (Fig. 3B). The maximum protein expression was estimated to be about 0.3% of soluble protein or about 1.44 µg per 1 g of leaf material. There are smaller molecular weight bands on the blot. They are products of proteolysis of the toxin occurring either in planta or during protein preparation. The expression of the protein product of the synthetic cry9Aa gene in tobacco

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Fig. 1. Synthetic cry9Aa gene sequence. The underlined nucleotides are different from the native gene sequence. Restriction sites dividing the gene in five fragments are shown in gray boxes. Arrows show size and direction of primers used in the DNA synthesis of the first fragment. Start codon is situated inside of NcoI restriction site. Stop codon TAA is situated between BglII and XhoI sites.

Fig. 2. T-region of the vectors used in transformation of plants with cry9Aa sequences. Abbreviations: 35S:Sp, double 35S promoter from CaMV; AMV, UTR leader from AMV; LB and RB, left and right borders of T-region of Ti plasmid; pA, polyadenylation signal site; hpt, hygromycin phosphate transferase gene; nos, nopalin synthase; p, promoter.
plants was at least 50 times higher than the sensitivity of our Western analysis, as the Cry9Aa protein signal was clearly detectable in a 50× dilution of the total protein of T-GS-1 transgenic line (Fig. 3C).

The truncated and truncated-GUS-fused constructs of native cry9Aa gene expressed in tobacco about ten times less of mRNA than the synthetic cry9Aa sequence. The average expression of the mRNA was 0.2–0.3 pg per 1 μg of total RNA (Fig. 4A and B). The GUS-fusion construct expressed in tobacco plants had two to three different size mRNA products: 4 kb is the entire sequence size and 0.8–2 kb fragments are degraded mRNAs (Fig. 4B). There was no clearly detectable Cry9Aa signal on the Western blot of the native gene constructs (Fig. 4C), indicating that the synthetic cry9Aa gene construct expressed in TGS-4 at least 50 times more protein than the native gene constructs.

3.3.2. Potato plants

Potato plants of cv. Pito were transformed with the synthetic cry9Aa gene construct. The Southern, Northern and Western analyses were performed using leaf tissue as source material. Plants positive in Southern analysis (data not shown) were examined in Northern analysis. Three transgenic lines (PGS-1, -2 and -8) expressed cry9Aa mRNA at a level of 3 pg per 1 μg of total RNA, the average expression being 2 pg/μg (Fig. 5A). Western analysis showed that line PGS-1 ex-
pressed Cry9Aa protein at a level of 0.03% of soluble protein or 300 ng per 1 g of leaf material, while the other lines had about 30–50% lower expression (Fig. 5B).

3.3.3. Brassica species

Cauliflower and turnip rape plants were transformed with synthetic cry9Aa gene. Plants positive in the Southern analysis were analyzed for their mRNA (Fig. 6A) and protein (Fig. 6B) expression. The Northern analysis showed that cauliflower line A-GS-0 expressed about 0.7 pg cry9Aa mRNA per 1 μg of total RNA, although high background made exact calculation difficult. Toxin protein expression of the line was about 0.01% of soluble protein or 100 ng per 1 g of leaf material. One cauliflower line A-GS-10, and two turnip rape lines (V-GS-12.1 and -14.3) expressed 0.1–0.2 pg of mRNA per 1 μg of total RNA. The corresponding protein products were undetectable on a Western blot.

3.4. Bioassays against P. xylostella

Transgenic cauliflower and turnip rape plants were assayed against P. xylostella. First plants were assayed in an 8-day feeding test in detached leaf bioassay. Preliminary tests showed that transgenic cauliflower (lines A-GS-0 and A-GS-10) and turnip rape plants (lines V-GS-12.1 and V-GS-
14.3) are 100% insecticidal to wild *P. xylostella* larvae (data not shown). The insecticidal effect, however, depended on the level of protein expression in the plants. The A-GS-0 line of cauliflower was highly toxic to *P. xylostella* larvae and killed the larvae in 2 days of feeding. This transgenic line killed also successfully both the CryIAc and the Cry1C resistant *P. xylostella* lines. The other transgenic line of cauliflower, A-GS-10, and two of the transgenic lines of turnip rape, V-GS-12.1 and -14.3, were also toxic to *P. xylostella* larvae, although less than A-GS-0. Preliminary 8-day cauliflower feeding tests (two to four repetitions of a feeding test with five larvae in each test, without daily monitoring of larval mortality) with the resistant lines of *P. xylostella* showed that the mortality of larvae ranged from 40 to 100% (Cry1C resistant line) to 100% (Cry1Ac resistant line). The highly expressing Cry9Aa cauliflower line effected 100% mortality to all of the wild type and Cry1C and Cry1Ac resistant lines of *P. xylostella*. To illustrate the insecticidal effect of a transgenic plant the feeding tests were monitored daily over a period of 14 days. The mortality and pupation of larvae were registered every second day. The results of the assay are shown in Table 1. To evaluate the insecticidal effect of the transgenic plants in extreme conditions the A-GS-0 and A-GS-10 transgenic lines of cauliflower were tested in another bioassay. The transgenic plants as well as the non-transgenic control plants were placed in a chamber with a high-density culture of *P. xylostella*. After 7 days of exposure the plants were photographed to register the leaf damage (Fig. 7). A plant of the A-GS-0 line had barely visible, very small traces of insect bites. The A-GS-10 line plant showed some damage, while the non-transformed control plants were entirely damaged by larvae.

### Discussion

The low expression level of the native *cry9Aa* gene in plants was a problem for its utilization for
insect control. Thus, truncations, modifications and finally complete synthesis of the sequence were carried out. Our truncated and truncated-GUS-fused constructs of the native \( \text{cry}9\text{Aa} \) gene expressed 20 times less mRNA than the synthetic gene in transgenic tobacco plants. The protein product of the native gene constructs was undetectable in Western analysis and was expressed at least 50 times less than the synthetic sequence. The GUS-fusion did not stabilize the native \( \text{cry}9\text{Aa} \) gene RNA or protein product in tobacco plants. Moreover, the mRNA product of the native \( \text{cry}9\text{Aa} \) gene clearly degraded into smaller molecules of 0.8–2.0 kb.

Our strategy for the modification of the \( \text{cry}9\text{Aa} \) gene was based on the principle of entire DNA sequence conversion from a bacillar to a higher plant gene. The codon preference was changed to resemble that of dicots in order to enhance the translation process. Then we removed all of the known putative mRNA processing sites from the gene sequence to enhance mRNA expression. Undesirable repetitions and restriction sites were also removed. In all, 24% of the nucleotide sequence was changed. All of the modifications in the gene sequence were performed without a single change in the amino acid sequence of the protein product.

There are reports confirming that rapid degradation of \( \text{cry} \) mRNA in plants is the main reason for the reduced expression of \( \text{cry} \) genes [32]. Moreover, rare codons are not sufficient to destabilize the expression level [33]. We have shown that the expression of the synthetic \( \text{cry}9\text{Aa} \) gene was higher than that of the native truncated gene by 20-fold at the mRNA level and at least 50-fold higher at the protein level. Because both of the genes were expressed under the control of the same promoter and containing the same polyadenylation sites, we conclude that the expression of a \( \text{cry} \) gene depends both on aberrant mRNA processing and on codon preference. Our data on expression of \( \text{cry}9\text{Aa} \) gene in plants are comparable with published reports [7,9,10]. Partial modification of the truncated native \( \text{cry}9\text{Aa} \) gene performed earlier [8] by site

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**Fig. 6.** Molecular analysis of \( \text{cry}9\text{Aa} \) gene expression in transgenic cauliflower cv. Asterix and turnip rape cv. Valitti. (A) Three \( \mu \)g of total RNA of cauliflower and turnip rape plants were loaded in the gel. The samples are shown as follows: C-RNA of non-transformed control plant, A-GS-0 and A-GS-10 lines of transgenic cauliflower. V-GS-12.1 and V-GS-14.3 are lines of transgenic turnip rape. Control RNA was loaded in the gel in series 12.5–2.5–5.0 pg. (B) Fifty \( \mu \)g of total soluble protein of cauliflower A-GS-0 and turnip rape V-GS-14.3 were loaded in the gel. A-C and V-V 50 \( \mu \)g of total protein of non-transformed cauliflower and turnip rape plants. Total protein of T-GS-1 transgenic line of tobacco containing 10 ng of Cry9Aa was mixed with 40 \( \mu \)g total protein of non-transgenic cauliflower line A-C and used as positive control.
Table 1

Transgenic cauliflower and turnip rape leaf-feeding bioassays against second instar larvae of *P. xylostella*, a susceptible wild *P. xylostella* line and lines resistant to CryIAc and CryIC toxins

<table>
<thead>
<tr>
<th><em>P. xylostella</em> Days of feeding (Line*)</th>
<th>2 day</th>
<th>4 days</th>
<th>6 days</th>
<th>8 days</th>
<th>10 days</th>
<th>14 days</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A-GS-0 line of cauliflower</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild</td>
<td>5 died</td>
<td></td>
<td>3 died</td>
<td>1 died</td>
<td>1 died</td>
<td></td>
</tr>
<tr>
<td>CryIAc</td>
<td>2 died, 3 alive</td>
<td>1 died, 2 alive</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CryIC</td>
<td>2 died, 3 alive</td>
<td>1 died, 2 alive</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>A-GS-10 line of cauliflower</strong></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Wild</td>
<td>5 alive</td>
<td>5 alive</td>
<td>3 died, 2 alive</td>
<td>2 died</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CryIAc</td>
<td>5 alive</td>
<td>5 alive</td>
<td>5 alive</td>
<td>5 alive</td>
<td>1 pupa, 4 alive</td>
<td>2 pupas, 2 died</td>
</tr>
<tr>
<td>CryIC</td>
<td>5 alive</td>
<td>5 alive</td>
<td>5 alive</td>
<td>5 alive</td>
<td>1 pupa, 4 alive</td>
<td>2 pupas, 2 died</td>
</tr>
<tr>
<td><strong>Control line of cauliflower</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>cv. Asterix</td>
<td>5 alive</td>
<td>5 alive</td>
<td>1 pupa, 4 alive</td>
<td>4 pupas</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild</td>
<td>5 alive</td>
<td>5 alive</td>
<td>2 pupas, 3 alive</td>
<td>3 pupas</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CryIAc</td>
<td>5 alive</td>
<td>5 alive</td>
<td>2 pupas, 3 alive</td>
<td>2 pupas, 1 alive</td>
<td>1 pupa</td>
<td></td>
</tr>
<tr>
<td>CryIC</td>
<td>5 alive</td>
<td>5 alive</td>
<td>2 pupas, 3 alive</td>
<td>2 pupas, 1 alive</td>
<td>1 pupa</td>
<td></td>
</tr>
<tr>
<td><strong>Turnip rape cv. Valtti</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V-GS-12.1</td>
<td>5 alive</td>
<td>2 died, 3 alive</td>
<td>3 died</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V-GS-14.3</td>
<td>1 died, 4 alive</td>
<td>2 died, 3 alive</td>
<td>2 died</td>
<td>4 pupas, 1 alive</td>
<td>1 pupa</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>5 alive</td>
<td>5 alive</td>
<td>5 alive</td>
<td>5 alive</td>
<td>5 alive</td>
<td>5 alive</td>
</tr>
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*Five larvae were used in each point of the experiment. Number of alive or dead larvae as well as the number of pupae are shown.*

directed mutagenesis increased expression efficiency, but, obviously, was not enough for high insecticidal effect [8]. Similar results were obtained from partial modifications of *cry1Ab* and *cry1Ac* genes [7]. Partial modifications mainly improve mRNA processing. This improvement can increase the expression level maximally by 10–20-fold according to our and earlier published [7] data. Complete resynthesis of a *cry* gene usually improves the expression by 100-fold [7,9,10]. In the case of *cry9Aa* the expression of a partially (4%) modified [8] gene can be evaluated only on the basis of the bioassay results. Gleave et al. [8] reported that potato tuber moth (*Phthorimaea operculella*) is sensitive to Cry9Aa toxin with an LC$_{50}$ value of 80 ng/ml, which corresponds approximately to the toxicity to *P. xylostella* in our bioassays (unpublished data). Comparing the bioassays data of the transgenic tobacco against potato tuber moth [8] and our transgenic cauliflower against diamondback moth, we can suppose that our plants express significantly more of Cry9Aa toxin than the tobacco plants of Gleave et al. [8]. However, the comparison is very approximate due to lack of data on expression of the gene. Our transgenic cauliflower killed all of the larvae in a 2-day feeding experiment, while the potato tuber moth mortality ranged from 50 to 100% in a 9-day feeding test on transgenic tobacco [8]. These data support the validity of our synthesis strategy for the *cry9Aa* gene.

The expression level we obtained of synthetic *cry9Aa* gene in tobacco and potato plants was close to the average level of expression of other genes driven by the CaMV 35S promoter. Transcription efficiency reached 3–5 pg of *cry9Aa* mRNA per 1 µg of total RNA, while the translation level varied between 0.03 and 0.4% of soluble protein corresponding to 0.3–1.4 µg of protein per 1 g of leaf material. Expression of the synthetic
A cry9Aa gene in cauliflower was measured to be 0.7 pg cry9Aa mRNA per 1 µg of total RNA and about 0.01% of soluble protein or 100 ng of Cry9Aa protein per 1 g of leaf material. While this expression level was 5–10 times lower than the level in tobacco or potato, it was still enough for a high insecticidal action. A drawback was that the expression was significantly lowered in mature transgenic Brassica plants. We argue, that reduction of cry9Aa gene expression in A-GS-0 transgenic cauliflower line occurred at the level of mRNA synthesis rather than at the level of translation, caused by blocking of the promoter action or by specific degradation of the cry9Aa mRNA. This is supported by the observation that silencing happened in the other transgenic Brassica plants, which still exhibited insecticidal traits. Our Northern analysis showed that these plants had 20–40 times less of cry9Aa mRNA compared to the transgenic tobacco plants. Unfortunately, because of the high background, Cry9Aa protein expression in turnip rape plants was not detectable in Western analysis (Fig. 6B). In spite of the loss in mRNA they retained the insecticidal effect and thus must have at least tens of nanograms of the cry9Aa toxin in 1 g of leaf tissue. The possibility that silencing is provoked by the double 35S promoter from CaMV or AMV leader from alfalfa mosaic virus can be checked by transforming the plants with the cry9Aa gene placed under the control of a promoter other than 35S. We also link the reduction in the expression level with the modified 35S promoter or the AMV leader we used in our study because there are reports on high cry1Ac [34] and cry1C [24] gene expression (0.4% of soluble protein) in canola and cauliflower driven by the non-modified 35S promoter. These data correlate with our own report on high level of GUS expression driven by the 35S promoter in turnip rape [23]. Nevertheless, the expression level of Cry9Aa protein in the transgenic cauliflower line, A-GS-0, was high enough to perform the insect bioassay, while the turnip rape plants expressed the cry9Aa gene only at a low level, but still revealed an insecticidal effect in bioassays.

Fig. 7. Bioassays of transgenic cauliflower plants against P. xylostella. The plants were placed into a chamber with a high number of P. xylostella insects. The picture was photographed after 1 week of feeding. The plants in the figure are as follows: transgenic cauliflower plants A-GS-0, moderate expressor and A-GS-10, a lower expressor of the synthetic cry9Aa gene. Non-transgenic cauliflower plants are shown on the upper part of the picture.
Overall, we report here a successful design and synthesis of an effectively expressing cry9Aa gene construct, which produced 20 times more mRNA and at least 50 times more protein product than the natural, truncated coding sequence. Expression of the synthetic cry9Aa gene in transgenic plants provides protection against several insect pests (P. xylostella, Pieris species, Phthorimaea operculella, Manduca sexta), which enables its use in crop protection programs.

References

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