Construction and rapid testing of synthetic and modified toxin gene sequences CrylA (b & c) by expression in maize endosperm culture

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Summary. The synthesis of two modified genes, CrylA(b) and CrylA(c), each consisting of 1845 bp, is described in detail. The genes were synthesized using an improved PCR procedure based on recursive principles. The synthetic CrylA(c) gene was put under the control of a maize ubiquitin promoter. This construct was tested in a maize endosperm-derived suspension culture system. The use of maize endosperm culture as a quick and efficient system to test the activity of synthetic genes is described.

Key words: Toxin genes - maize endosperm culture - PCR - Ubiquitin promoter

Introduction

We are interested in engineering crops (e.g. tropical maize, rice) and tree species with the modified endotoxin genes. Both the CryIA(b) and CryIA(c) endotoxins have been shown to be effective against the major insect pests of maize and rice. In previous studies, only the modified CryIA(b) gene has been introduced into maize and rice (two different modified versions; one with G+C content of 65% designed to match maize codon usage and the other with G+C content of 59.2% to match codon preference in rice (Fujimoto et al. 1993; Koziel et al. 1993). Fully modified (FM versions) CrylA(b) and CrylA(c) genes (1845 bp truncated) have been previously reported to be highly expressed in dicots (Perlak et al. 1990; Perlak et al. 1991). The details and procedures for the complete synthesis of these same two modified genes have not been described. In terms of codon usage, these specific genes were designed for expression in dicots and had G+C content of 49% for CryIA(b) gene and 47.7% for crylA(c) gene. The original genes had a G+C content of 37%. The overall modifications resulted in 100-fold increase in their expression in dicot transgenic plants.

These particular sequences (the FM versions with G+C content of 49% & 47.7%; see Perlak et al. 1990; Perlak et al. 1991) have not been tested for expression in monocots so we set out to build the exact same sequences for modified CrylA(b) and CrylA(c) toxin genes. The long term objective would be to acquire important information about deciding whether to design modified genes for every different plant species.

We synthesized the modified genes using an improved PCR procedure. This procedure is based on recursive principles (Prodromou and Pearl 1992). To our knowledge, this is the first detailed report of the totally PCR-directed synthesis of two large synthetic and modified CrylA(b) and CrylA(c) bacterial toxin genes. In other studies, two different modified CrylA(b) genes were synthesized but detailed reaction conditions were not reported (Fujimoto et al. 1993; Koziel et al. 1993). Another B.t. toxin gene, CylIIA in the similar size range was synthesized using ligation approach methods (Adang et al. 1993; Sutton et al. 1992).

Traditionally, the testing of synthetic and modified B.t. toxin genes has required the use of plant protoplasts and/or transgenic plant systems (Adang et al. 1993; Fujimoto et al. 1993; Koziel et al. 1993; Perlak et al. 1991; Sutton et al. 1992). Such systems are time-consuming and difficult simply in terms of "assaying" though on a long term basis stable transgenic plant systems have their advantages. Unfortunately, the monocots such as rice and maize are even more difficult and
laborious to transform.

With the increasing need for testing different versions of the same or different modified toxin genes in different plant species, it has become necessary to develop more rapid, efficient and direct B. t. gene expression assays. We report on the use of a maize endosperm-derived suspension culture in combination with the maize ubiquitin promoter as an alternative, rapid and effective way to test the expression of modified B.t. toxin genes. This procedure is more direct and does not require the isolation of protoplasts which is cumbersome and laborious. Previously, isolated protoplasts from such cultures have been used mainly to study maize storage protein genes and promoters (Quayle et al. 1991; Ueda and Messing 1991).

This is the first report of the activity of the ubiquitin promoter and synthetic CryIA(c) gene in maize endosperm-derived suspension culture. This ubiquitin promoter has been tested before in protoplasts from the BMS maize line and tobacco TXD cell culture (Christensen et al. 1992) and transgenic rice plants (Cornejo et al. 1993; Toki et al. 1992). We have used this promoter for the rapid testing of a synthetic and modified CryIA(c) gene in a monocot. The same modified CryIA(c) gene (under the control of CaMV 35S promoter) has been tested earlier only in dicot plants (Perlak et al. 1990; Perlak et al. 1991).

Materials and methods

Plant material. The maize endosperm-derived suspension culture from inbred line A636 was obtained from Dr. Jack Shannon and maintained and subcultured every seven days according to the procedures described earlier (Shannon 1982; Shannon and Liu 1977). The suspension cells were collected and cultured on agar medium (Shannon 1982) for two days before bombardment.

The oligos and PCR conditions. A majority of the oligos were 60-69 mers (Synthaid Biotechnologies, Inc., Nepean, Ont.). The first phase PCR reaction contained 1 picomole of each of the oligos (representing almost entirely the upper and lower strands of a particular block), 10 µl of 10X Vent buffer, 250 µM of each of the dNTPs, 6mM MgSO4 and two units of Vent polymerase (New England Biolabs). The PCR conditions were one minute at 94°C, one minute at 55°C and one minute at 72°C. The first cycle differed from subsequent cycles by having a longer denaturation time of 5 minutes. One final extension of five minutes was used to close the first phase. The second phase amplification was performed on a 5.0 µl aliquot (from the first phase) using outer oligos. The other PCR conditions and parameters were the same as for the first phase.

DNA cloning, sequencing and constructs. The cloning and assembly of the genes was done using standard procedures (Sambrook et al. 1989) and kits from commercial manufacturers.

Particle bombardment and CUS assay. The suspension cells were bombarded using the PDS-1000/He particle delivery system (Bio-Rad) according to the established procedure (Klein et al. 1987). The plumps of cells were bombarded with DNA-coated gold particles (1.0 mg particles coated with 5 µg of DNA) at a pressure of 1300 psi. The plate distance was approximately 12.5 cm from the stopping screen. The bombarded samples were stained with X-Gluc as described before (Jefferson et al. 1987).

Isolation of proteins and Western blot analysis. Proteins were isolated as previously described (Koziel et al. 1993). Briefly, 100 mg of the pooled tissue was ground in 100 µl of the extraction buifer. The sample was vortexed and spun for 5 minutes in a microcentrifuge. A 10 µl aliquot was subjected to Western blot analysis. The proteins were separated in 10% polyacrylamide gel containing SDS (Laemmli 1970). After electrophoresis, the proteins were electrotransferred onto nitrocellulose membrane using an electrotransfer apparatus according to the manufacturer’s instructions (Bio-Rad). The blocking was done for an hour with 5% non-fat dry milk in TBS buffer with 0.05% Tween 20 (Promega). Incubations with primary antibody [rabbit anti-CryIA(b) antisera (0.2 mg/ml) from CIBA-GEIGY; 1:1000 dilution] in blocking solution were for 2 hours. The membrane was washed three times in a buffer (TBS with Tween 20). It was then probed with alkaline phosphatase-conjugated anti-rabbit IgG (1:3000 dilution; Promega) by addition of nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt (GibcoBRL) in alkaline phosphatase substrate buffer (Promega). The sample event 176b (Koziel et al. 1993) represents the leaf material of a B.t. toxin producing transgenic maize plant from the Ciba-Geigy Corporation, North Carolina.

Results and discussion

Construction of synthetic toxin genes

For the synthesis of toxin sequences, we developed an improved PCR procedure and it is based on previously described recursive principles (Prodromou and Pearl 1992). The new protocol involves a two-phase PCR reaction compared to the one step approach described earlier. A slow cool step was also introduced in PCR synthesis to improve the product quality.

Each of the two genes assembled from blocks, referred to as E, MI, MII, Sb and Sc, is shown in Fig. 1.

<table>
<thead>
<tr>
<th>BankI</th>
<th>XbaI</th>
<th>XbaI AcoI</th>
<th>AcoI SstI</th>
<th>SstI EcoRI</th>
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<tr>
<td>668 bp</td>
<td>403 bp</td>
<td>279 bp</td>
<td>495 bp</td>
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SD SstI EcoRI

Fig. 1. The five blocks used for synthesis and assembly of CryIA(b) and CryIA(c) truncated and modified genes of endotoxins from Bacillus thuringiensis. The blocks E, MI, MII, Sb and Sc of sizes 668, 403, 279, 495 and 495 bp respectively are shown here along with the desired restriction sites. There were 22 oligos used for block E, 14 for MI, 12 for MII, 16 for Sb and 18 for Sc. For each oligo the number of sense and antisense oligos were the same.

The three blocks E, MI and MII were common for the two genes. The blocks Sb and Sc represent the specific parts unique to the CryIA(b) and CryIA(c) genes respectively.