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Characterisation of synthetically developed *cry1Ab* gene in transgenic tobacco chloroplasts

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Abstract: Synthetic *cry1Ab* gene was developed with optimised codons and expressed in the tobacco plastome. Characterisation of the synthetic *cry1Ab* gene was carried out on homoplasmic transgenic shoots. To purify the homoplasmic shoots, the proliferating heteroplasmic shoots were subjected to various rounds of selection and regeneration on spectinomycin-containing regeneration medium of plant medium. The PCR-screened homoplasmic shoots were placed on MS medium for root induction and proliferation. The functionally expressed CRY protein was detected using ImmunoStrips, and the amount of toxin protein in transplastomic tobacco plants was measured by DAS-ELISA, specific for *cry1Ab* gene product. The accumulation of *Bacillus thuringiensis* (*Bt*) toxin protein in transgenic chloroplasts confirmed that the synthetic gene could be expressed in other crop plants using constitutive and tissue specific regulatory sequences.

Key words: Characterisation, synthetically developed *cry1Ab* gene, transgenic chloroplasts

1. Introduction

The chloroplast genome of plant cells has become amenable to genetic engineering (Daniell et al., 2002; Zerges, 2004; Bock & Khan, 2004). Chloroplast genetic engineering offers several unique advantages, including high-level transgene expression, multigene engineering in a single transformation event, and transgene containment by maternal inheritance (Khan, 2005). The process relies on the homologous recombination machinery of the plastid, incorporating foreign DNA into the plastome (Khan et al., 2005), and results in uniform gene expression eliminating position effects and epigenetic gene silencing (Daniell et al., 2002) often observed among nuclear transgenic plants. Agronomically important traits, such as improved tolerance to salts, drought, herbicide, and resistance to pathogens and insects, have been engineered in the plastid genome with success, demonstrating how this technology is environmentally friendly and a promising tool for biotechnologists (Daniell et al., 1998; Kumar et al., 2004; Craig et al., 2008; Zhang et al., 2008).

The development of insect resistance in plants is an important aspect in augmenting food security, as insects cause huge loss to crops and plants of agronomic importance. *Bacillus thuringiensis* (*Bt*) insecticidal genes (*cry* genes specifically) have been in use for over 3 decades for the control of Lepidopteran, Dipteran, and Coleopteran insect pests (Schnepf et al., 1998). The *cry*-toxin genes are difficult to express at higher levels in the nucleus even under the control of efficient plant regulatory sequences because prokaryotic genes could not be efficiently expressed in the eukaryotic system of expression. The most likely solution to this is to express these genes in plastids. There is direct evidence of rapid degradation of the native *Bt* toxin mRNA under the nuclear system of expression (De Rocher et al., 1998). In addition, most insects (i.e. caterpillars) feed on green tissues rich in chloroplast, and therefore would consume highest levels of insecticidal toxins if these are expressed in the chloroplast. In the present study, the plastidic expression of the codon optimised *cry1Ab* gene confirms that the synthetic *cry1Ab* gene could be expressed in other crop plants using constitutive and tissue specific regulatory sequences (Oğraş et al., 2000).

2. Materials and methods

2.1. Resynthesis of codon optimised *cry1Ab* gene

Resynthesis of codon optimised *cry1Ab* gene was carried out using overlapping oligos. The detailed sequence of the gene along with engineered UTR and the restriction sites are described in the results. However, the transformation vector was briefly described by Jabeen et al. (2009).

2.2. Tobacco chloroplast transformation using the biolistic method

The juvenile leaves of aseptically grown plantlets were used for the regeneration of plantlets (Verma et al., 2011) through

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the transformation procedures. The transformation procedures were carried out essentially as described by Svab and Maliga (1993). Gold particles from BioRad (1 µm diameter) coated with developed transformation vector DNA were used for the chloroplast transformation of tobacco leaves following the standard chloroplast transformation protocol (Khan & Maliga, 1999). Two days after bombardment, the leaves were chopped into small pieces and placed on the same regeneration medium (Svab & Maliga, 1993; Khan & Maliga, 1999; Khan et al., 2007) containing spectinomycin (500 mg/L).

The regenerated resistant shoots were chopped into small pieces (about 2 mm) and subcloned into fresh deep petri plates containing the same selection medium for other rounds of selection and regeneration to purify homoplasmic shoots. The homoplasmic shoots were proliferated for root induction on spectinomycin-containing MS medium (Murashige & Skoog, 1962) in sterile glass bottles under controlled conditions at 27 °C for 7 days (Usman et al., 2008).

2.3. *Cry1Ab/1Ac* ImmunoStrip test

The *cry1Ab/Ac* specific strips (Agdia, Catalogue # STX 06200) were used to determine the expression of *cry1Ab* protein in transplastomic plants following the instructions of the manufacturer.

2.4. *Cry1Ab/1Ac* DAS-ELISA test

Agdia PathoScreen kit for *Bt-cry1Ab/1Ac* protein peroxidase (label Catalogue # PSP 06200) was used to estimate the amount of *cry1Ab* toxin protein in tobacco leaves following the standard Agdia protocol for DAS-ELISA.

3. Results

3.1. Synthesis of *cry1Ab* gene, vector construction and chloroplast transformation of tobacco leaves

The synthesis of *cry1Ab* gene, vector construction, and chloroplast transformation of tobacco leaves were performed as follows. The bacterial *cry1Ab* gene was codon optimised by designing overlapping oligos, taking into consideration the codon preference of cereals for high expression (Perlak et al., 1991; Carozzi et al., 1992; Fujimoto et al., 1993; Koziel et al., 1996; Sardana et al., 1996; Cheng et al., 1998). The *cry1Ab* gene of 1932 bp was resynthesised by assembling the oligos in PCR reactions following the manufacturer's instructions (Fermentas, Germany) and the gene was sequence confirmed (Figure 1). Later on, a downstream box (5'-UTR along with the N-terminal sequence from the *psbA* gene) was amplified to enhance the gene expression in chloroplasts (Khan & Maliga, 1999; Bock & Khan, 2004). Since the *cry1Ab* gene is GC rich, it is preferably expressible to high levels in the eukaryotic system. A fragment of 1994 bp (including restriction sites, plastid-specific sequence, and *cry1Ab* gene) cloned into the pTZ57RT vector was used to develop the final chloroplast transformation vector as described (Jabeen et al., 2009). The expression cassette was cloned into the final transformation vector downstream of the *aadA* gene as shown in the physical map (Figure 2). The *aadA* gene that confers resistance to spectinomycin was used in the selection of transgenic plants (Svab & Maliga, 1993; Khan & Maliga, 1999; Liu et al., 2008).

3.2. Genomic analysis of spectinomycin-resistant tobacco clones

Spectinomycin-resistant clones regenerated from bleached leaf sections were subjected to genomic analysis and antibiotic-resistant *cry1Ab* positive clones, along with spontaneous spectinomycin-resistant shoots, were detected (Jabeen et al., 2009). These clones are chloroplast transgenic plants because *cry1Ab*, under the chloroplast-specific promoter, is known not to express in the nucleus; moreover, high level of spectinomycin in the medium allows only transplastomic lines to proliferate and not of nuclear ones because of low expression in the nucleus. One of the reasons for low expression in the nucleus are the ambiguity of foreign DNA integration in the heterochromatin region. Additionally, promoters are designed to be plastid specific rather than of the eukaryotic type. Heteroplasmic shoots were subjected to further rounds of selection and regeneration on spectinomycin-containing RMOP medium (Figure 3a) and the regenerated shoots were transferred to MS medium for root induction and proliferation (Figure 3b).

3.3. Protein analysis of transgenic chloroplast lines

PCR confirmed homoplasmic plants were subjected to protein analysis using ImmunoStrips and DAS-ELISA specific for *Cry1Ab*/*Cry1Ac* gene products. Two bands appeared on each ImmunoStrip dipped into the extracts from transgenic plants as compared to the control where a single band appeared (Figure 4). The first or upper band represents the control to confirm the validity of the *Bt cry1Ab/1Ac* ImmunoStrip test procedure, and the lower band on the strips indicates the expression of the *cry* gene in chloroplasts as confirmed by the quantification of expressed *cry* toxin protein through DAS-ELISA (Figure 5). In ELISA, the enzyme conjugate (consisting of an antibody chemically linked to an enzyme) links to the captured BT-Cry1A protein through its antibody portion while its enzyme part reacts with the substrate and gives the blue coloration upon the addition of the tetramethylbenzidine (TMB) substrate, signifying the presence of CRY1Ab protein. Total cellular proteins were detected using the Bradford assay for calculating the toxin in the leaves. An amount of 45 to 46 ng/mg toxin of fresh weight was calculated; nevertheless, higher levels of the toxin proteins were detected when the downstream box was removed and the gene expressed under the tissue-specific promoter in cereals (Khan et al., 2010). However, these results depict that the resynthesised gene is expressible in crop plants using various promoters.

*Nde*I catatg.....

ATGGATAACAATCCGAACATCAAcGAgTGCATcCCcTAcAACTGccTGAGcAACCCcGAG 60

GTgGAgGTgcTgGGcGGcGAgcGcATcGAgACcGGcTAcACCCCcATCGAcATcagCcTG 120

agcCTgACcCAgTTcCTgcTGAGcGAgTTcGTgCCCGGcGCcGGcTTcGTGcTgGGcCTg 180

GTgGAcATcATcTGGGGcATcTTcGGcCCCagcCAgTGGGACGCcTTcCTgGTgCAgATc 240

GAgCAGcTgATcAACCAgcGcATcGAgGAgTTCGCccGcAACCAgGCCATcagccGccTg 300

GAgGGcCTgAGCAAcCTgTAcCAAATcTACGCcGAgagcTTccGcGAGTGGGAgGCcGAc 360

CCcACcAAcCCcGCccTgcGcGAgGAGATGCGcATcCAgTTCAAcGACATGAACAGcGCC 420

CTgACcACCGCcATcCCcCTgTTcGCcGTgCAgAAcTAcCAgGTgCCcCTgcTgagcGTg 480

TAcGTgCAgGCcGCcAAccTgCAccTgagcGTgcTGcGcGAcGTcagcGTGTTcGGcCAg 540

cGcTGGGGcTTcGAcGCCGCcACcATCAAcAGcCGcTAcAACGAccTgACccGcCTgATc 600

GGCAACTAcACcGAcCAcGCcGTgCGCTGGTACAAcACcGGccTgGAGCGcGTgTGGGGt 660

CCcGAcagccGcGAcTGGATcAGgTAcAAcCAgTTccGccGcGAgcTgACcCTgACcGTg 720

cTgGAcATCGTgagcCTgTTcCCcAACTAcGAcAGccGcACcTAcCCcATcCGcACcGTg 780

agCCAgcTgACccGcGAgATTTAcACcAACCCcGTgcTgGAgAAcTTcGAcGGcAGcTTc 840

CGcGGCagcGCcCAGGGCATcGAgGGcAGcATccGcAGcCCcCAccTGATGGAcATcCTg 900

AACAGcATcACCATCTAcACcGAcGCcCAccGcGGcGAgTAcTAcTGGagcGGcCAcCAg 960

ATcATGGCcagcCCcGTcGGcTTcagcGGcCCcGAgTTCACcTTcCCcCTgTAcGGcACc 1020

ATGGGAAATGCAGCTCCACAACAACGTATTGTTGCTCAACTAGGTCAGGGCGTGTATGGA 1080

ACccTgagcagCACccTgTAccGTcGACCTTTcAACATcGGcATcAACAACCAgCAgCTg 1140

agcGTgCTgGACGGcACcGAgTTcGCcTAcGGcACCagCagcAAccTGCCcagCGCcGTg 1200

TACcGcAAgAGCGGcACcGTgGAcagcCTGGAcGAgATcCCcCCtCAGAAcAACAACGTG 1260

CCACCTcGaCAgGGcTTcAGcCAcCGtcTgAGCCAcGTgagcATGTTcCGcagtGGCTTc 1320

AGcAACAGcAGcGTgAGcATcATccGtGCaCCTATGTTCagcTGGATtCAcCGcAGTGCc 1380

GAgTTcAACAACATcATcCCcagcagcCAAATcACcCAgATcCCccTgACcAAgagcACc 1440

AACCTgGGCagcGGcACcagcGTgGTgAAgGGcCCcGGcTTcACcGGcGGcGAcATcCTg 1500

CGccGcACcagccCcGGCCAGATcagcACCcTgcGcGTgAAcATcACcGCcCCccTgagc 1560

CAgcGcTAcCGcGTccGcATcCGCTACGCcagcACCACcAAccTgCAgTTCCAcACcagc 1620

ATcGACGGccGcCCcATcAAcCAGGGcAAcTTcagcGCcACcATGAGcAGcGGcAGcAAc 1680

cTgCAGagCGGcAGCTTccGcACcGTgGGcTTcACcACcCCcTTcAACTTcagcAAcGGc 1740

agcAGcGTgTTcACccTgAGcGCcCAcGTgTTCAAcagcGGCAAcGAgGTgTAcATcGAc 1800

CGcATcGAgTTcGTgCCcGCcGAgGTgACCTTcGAGGCcGAgTAcGAccTgGAGAGgGCt 1860

CAgAAGGCcGTGAAcGAGCTGTTcACcagcagCAAcCAgATCGGccTgAAgACcGATGTG 1920

ACcGAcTAgCAc..... tctaga *Xba*I

Figure 1. Nucleotide sequence of the chemically resynthesised *cry1Ab* gene developed using modified codon sequences (Perlak et al., 1991; Carozzi et al., 1992; Fujimoto et al., 1993; Koziel et al., 1996; Sardana et al., 1996; Cheng et al., 1998). Small letters indicate the optimised codons and dashed lines present sequences other than *Bt* gene as referenced in the text.

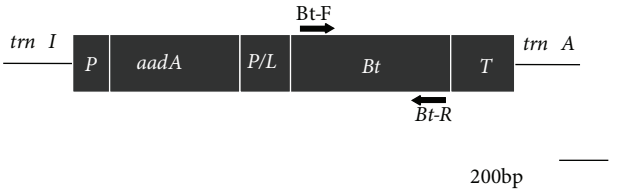

Figure 2. Map of the transformation vector with primer positions for the introduction of the resynthesised *cry1Ab* along with the marker gene into the chloroplast genome of tobacco. Shown are: P, plastid ribosomal RNA promoter; p/L, plastid ribosomal RNA promoter along with *psbA*UTR *aadA*, aminoglycoside 3"-adenyltransferase; *Bt*, *Bacillus thuringiensis*; T, terminator; Bt-F and Bt-R are *Bt* gene specific forward and reverse primers, respectively.

4. Discussion

One of the most promising ways to reduce dependence on pesticides in agriculture is to develop insect-resistant crops. The initial attempts to generate insect resistant plants through the expression of *Bt* toxin genes in the nucleus resulted in very low expression levels due to a number of reasons such as mRNA instability, differences in codon usage, and aberrant transcript splicing (Perlak et al., 1991; Simpson & Filipowicz, 1996; Cogoni & Macino, 1997). In this context, subsequent attempts focused on the construction of synthetic gene versions to avoid these undesirable features. The sequence was adjusted to the codon usage in the plant nucleus and hence expression was dramatically improved (Perlak et al., 1991; Adang et al., 1993; Koziel et al., 1996). This, together with the testing of a variety of promoters and other sequences, led to a significant improvement in insect control and *Bt* toxin levels to a maximum of 0.8% of the total leaf protein, expressed through nuclear transformation (Wong et al., 1992). However, high *Bt* toxin levels were achieved when the gene was expressed in chloroplasts (McBride et al., 1995) or when the gene product was targeted to plastids (Kim et al., 2009).

Taking into consideration the GC/AT content ratio (in codon preference) of cereals, the synthetic *cry1Ab* gene was further codon optimised and hence 2 genes (1 with 64% and the other with 55% GC content) were developed. A gene with 64% GC content (Figure 1) was introduced into the tobacco plastome in the inverted repeat region under the chloroplast-specific promoter to examine the expression levels. The signals that appeared on the ImmunoStrips indicate that a codon optimised gene is expressible in chloroplasts. Though the cassette was targeted to the inverted repeat region of the plastome, expression was, nevertheless, comparatively low. This was due to the heteroplasmic nature of plants and because of the high GC content of the gene, expressible to high levels in the eukaryotic system. Later on, purified homoplasmic lines showed comparatively high levels of gene expression but were not of the levels normally achieved under chloroplast expression elements. Therefore, it was concluded that the synthetic *Bt* gene with a high GC content can be expressed from the nuclear genome using eukaryotic regulatory sequences. Nevertheless, other version of the gene with low GC content produced high levels of toxin (Khan, MS, unpublished). However, the site-directed integration of foreign DNA in chloroplasts avoids position effects that are common in nuclear transformation, and that cause undesirable variations in gene expression (Daniell et al., 2002). Secondly, high polyploidy of the chloroplast genome in the cell also augurs well for such high expression (Bock & Khan, 2004). However, these studies conclude that similar synthetic genes could be codon optimised (depending upon the plant genotype) for expression in organelles successfully.

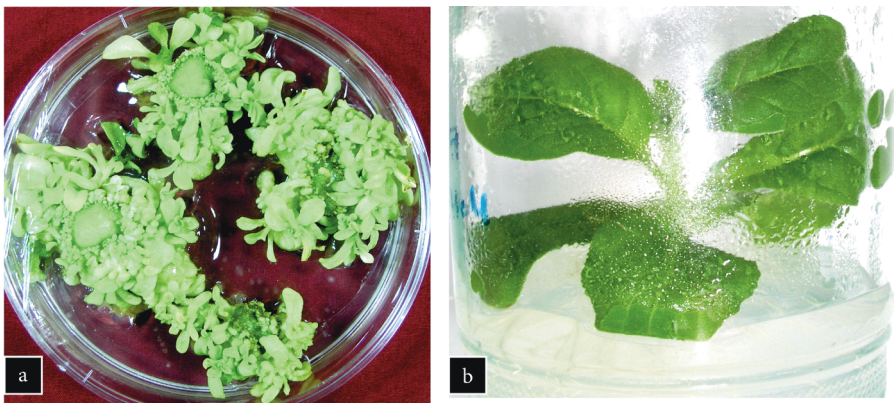

Figure 3. Regeneration of spectinomycin-resistant plants. a- Leaf sections from heteroplasmic shoots were subjected to a further round of selection and regeneration to purify homoplasmic shoots on spectinomycin-containing RMOP medium. b- Regenerated homoplasmic shoot is proliferated on spectinomycin-containing MS medium to develop roots.

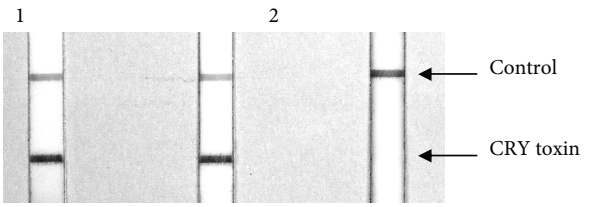

Figure 4. Qualitative analysis of transgenic plants for *Bt* toxin. ImmunoStrip test was performed to screen the plants on the basis of the expression of *Bt* toxin in chloroplasts. Lanes 1 and 2 represent transgenic plants, whereas lane 3 represents the wild-type, nontransgenic plant. The upper band represents the control reaction, whereas the lower band represents *Bt* toxin in the transplastomic plants.

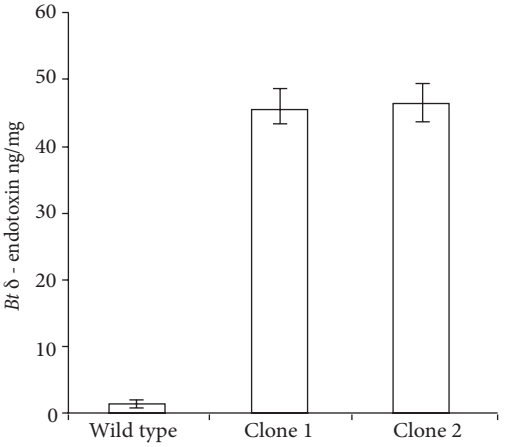

Figure 5. Quantitative expression of synthetically developed *Bt* gene in tobacco chloroplasts. Bar 1 indicates the nontransformed wild-type, whereas bars 2 and 3 indicate transplastomic clones 1 and 2 of the tobacco plant, respectively.

The method of deployment of crop plants expressing *Bt* toxins has been debated due to the likely build-up of resistant insect populations. One attractive proposal for insect resistance management is the "high dose strategy" (McBride et al., 1995). This method requires nearly 100% mortality of insects heterozygous for a resistance allele and the maintenance of nontransformed plants to insure that insects homozygous for a resistance gene are greatly outnumbered by and mate with susceptible insects (Rotish, 1994). The high level of *Bt* toxin produced in plastids is well suited to this strategy since resistance alleles in the target insects are less likely to provide protection against *Bt* transformed plants. Bilang and Potrykus (1998) have discussed requirements for transforming chloroplasts of agronomically useful crops such as cotton, maize, wheat, and sugarcane. One of the major limitations is in locating spacer regions (between any 2 genes) and transcriptional units to target site-specific integration of foreign genes. To overcome this limitation, universal vectors have also been developed (Daniell et al., 1998) that can transform any chloroplast genome due to its ability to integrate into the highly conserved region. Yet another advantage of expressing insecticidal proteins in chloroplasts is tissue specificity. Lepidopteran insects mostly feed on green leaf tissues that are rich in chloroplasts, thereby consuming the highest level of insecticidal protein (Kota et al., 1999). The high expression of foreign genes in chloroplasts is devoid of any detrimental or pleiotropic effects on plant growth (Daniell et al., 2002), physiological relations, and reproduction. Nevertheless, further studies are required since these were preliminary studies to test the expression levels of the genes, and to investigate the expression levels and insect control after expressing the gene versions from the nucleus as well as the chloroplast in the genomes of cereals.

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