Development and Evaluation of the Cotton Leaf Curl Kokhran Virus-Burewala Bidirectional Promoter for Enhanced Cry1Ac Endotoxin Expression in Bt Transgenic Cotton

Muhammad Aleem Ashraf 1,2,3,*, Ahmad Ali Shahid 1, Abdul Qayyum Rao 1, Judith K. Brown 2, and Tayyab Husnain 1

1 Centre of Excellence in Molecular Biology, University of the Punjab, Lahore 53700, Pakistan
2 School of Plant Sciences, The University of Arizona, Tucson, AZ 85721, USA
3 Institute of Biological Sciences, Faculty of Natural and Applied Sciences, Khwaja Fareed University of Engineering and Information Technology, Rahim Yar Khan 64200, Pakistan

* Correspondence: aleem.ashraf@kfueit.edu.pk; Tel.: +92-333-6346277

Abstract: Fluctuation in Cry1Ac endotoxin levels expressed in transgenic Bacillus thuringiensis (Bt) cotton (Gossypium hirsutum L.) can result in a variation in efficacy throughout the growing season. Here, a green tissue-specific strong promoter of the cotton leaf curl Kokhran virus-Burewala (CLCuKoV-Bu) C1 gene is reported that can direct consistently high levels of Cry1Ac endotoxin expression in transformed cotton plants. The objective of this study was to investigate the capacities of the CLCuKoV-BuC1 promoter to drive transcription of Cry1Ac and stably express endotoxin in mature leaves and bolls of transgenic cotton plants, compared to the traditional CaMV35S promoter. The Cry1Ac gene expression cassettes were constructed under the control of a bidirectional promoter and transformed into cotton ‘MNK-786’. The expression of Cry1Ac constructs was evaluated in transient and stable expression systems using Nicotiana tabacum ‘Rustica’ and cotton plants, respectively. Accumulation of the Cry1Ac expressed in two resultant transgenic cotton plants harboring the constructs driven by the CLCuKoV-BuC1 and CaMV35S promoter, respectively, was analyzed using a commercially available enzyme-linked immunosorbent assay. In leaves and bolls of two cotton plants shown to express CLCuKoV-BuC1-Cry1Ac (CLCuV-Ac), the Cry1Ac protein accumulated at 400 and 300 ng g⁻¹ per fresh tissue weight, respectively, whereas no toxin was detectable in the roots. In contrast, CaMV35S-Cry1Ac transgenic cotton plants accumulated three times less Cry1Ac protein than those transformed with CLCuV-Ac. Results indicate that the greatest amount of Cry1Ac endotoxin accumulated in transgenic cotton when expression was driven by the CLCuKoV-BuC1 compared to the traditional CaMV35S promoter. The newly validated CLCuV-Ac promoter of begomoviral origin offers an exciting alternative as a robust promoter for genetic engineering of cotton and other plants.

Keywords: Bacillus thuringiensis; Cry1Ac Endotoxin; cotton transformation; CLCuKoV-BuC1 promoter; gene constructs

1. Introduction

Cotton (Gossypium hirsutum L.) is cultivated in more than 50 countries worldwide and the cotton crop is a major component of the economy of Pakistan as a highly valued cash crop [1]. Cotton cultivation in Pakistan faces a number of problems, such as insect pests, plant viruses and weeds. Traditionally, insect pests have been controlled by agrochemicals, but their widespread use poses human health and economic risks. In recent decades, the development of transgenic crops has created opportunities for major biotechnological breakthroughs in solutions to pest and pathogen management [2,3]. Alternatives to agrochemicals, genetically modified (GM) crops expressing cry alleles (Cry1Ac, Cry1Ab, Cry2A...
and Cry2Ab) isolated from Bacillus thuringiensis (Bt) are grown widely on all continents [4–7]. Spatio-temporal variations in Bt-Cry1Ac endotoxin levels have been especially observed in transgenic cotton during the late stages of growth and development [8,9]. Consistent expression levels of Bt-Cry1Ac endotoxin in cotton plants throughout the growing season is essential, particularly in the most vulnerable organs and tissues to achieve sustainable management of insect pests including lepidopterans that cause extreme damage to cotton plants. The Bt-Cry1Ac endotoxin has been reported to occur in higher levels in transgenic plants early in the season, compared to late-season cotton growth and mid to late-season development stages [10].

Geminiviruses serve as a potential source of bidirectional gene promoters, regulatory enhancers, and cis-acting as well transcriptional regulatory elements that can be used to enhance transgene expression in transgenic plants [11,12]. Additionally, geminiviral repliscons can be used as viral vectors for plant genome-editing objectives [13,14]. Previously, the geminivirus large intergenic region (LIR) has been explored as a gene targeting for homologous recombination in plant genome engineering [15–18]. The DNA-A genome of monopartite begomoviruses possesses a LIR sequence that functions as a bidirectional promoter, essential for replication and expression of the viral genes, C1 (replication associated protein) and V1 (coat protein) [19,20]. Recently, Cry1Ac endotoxin expression in plants has been driven by plant promoters including the rbcS [10,21], AoPR1 [22,23], ats1A [24,25], and maize ubiquitin-1 promoters [26].

The objective of this study was to develop a local cotton variety that expresses the Bt-Cry1Ac gene in green tissues only and at consistent levels throughout the growing season. For this purpose, a robust promoter from the ‘Burewala’ strain of CLCuKoV was selected and characterized [19] prior to evaluating its ability to drive the expression of Bt-Cry1Ac gene. The expression cassettes containing Cry1Ac gene driven by the CLCuKoV-Bu bidirectional promoters were constructed. Transient expression of Cry1Ac promoter was evaluated initially in Nicotiana tabacum L. ‘Rustica’ after which the constructs were transformed into cotton ‘MNH-786′ using Agrobacterium-mediated transformation. The goal was to utilize the begomoviral bidirectional promoter to drive robust, stable, consistent expression of the Bt-Cry1Ac gene in transgenic cotton plants.

2. Materials and Methods

2.1. Biological Materials

Tobacco N tabacum ‘Rustica’ plants were grown for eight weeks in a composite soil in the CEMB greenhouse and used for agroinfiltration experiments to evaluate transient expression. The expression cassettes were transformed into competent E. coli (DH5α and TOP10) cells and Agrobacterium tumefaciens strains GV1301 and LBA4404. A full-length clone of the CLCuKoV-Bu and the PIA-2 plasmid vector Cry1Ac+NOS under control of the ubiquitin promoter was available in the laboratory. The binary plasmid vector pCAMBIA1301 harboring the begomoviral promoter was evaluated for transient expression in Nicotiana tabacum. Hygromycin (hyt) and kanamycin- resistance genes were used as selectable markers for plant and bacterial selection, respectively. The PK2-Ac plasmid the Cry1Ac+NOS insertion driven by the CaMV35S promoter was used as the positive control (Table 1).
### Table 1. Bacterial strains, viral species, plasmid vectors and plant species.

<table>
<thead>
<tr>
<th>Biological Materials</th>
<th>Relevant Features</th>
<th>Reference or Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacterial Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LBA4404 and GV1301</td>
<td>Agrobacterium tumefaciens strain used for plant transformation</td>
<td>[27]</td>
</tr>
<tr>
<td>DH5α</td>
<td>Escherichia coli strain used for cloning</td>
<td>[28]</td>
</tr>
<tr>
<td>TOP10</td>
<td>Escherichia coli-based cells used for cloning</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>CEMB-Cry1Ac</td>
<td>Bacillus thuringiensis δ-endotoxin gene Cry1Ac</td>
<td>CEMB Patent: (K140649-aroA)</td>
</tr>
<tr>
<td><strong>Viral Species</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CLCuKoV-Bu-Bu</td>
<td>Circular, single-stranded, DNA-A genome of the cotton leaf curl Kokhran virus-Burewala</td>
<td>[29]</td>
</tr>
<tr>
<td>LIR</td>
<td>Large intergenic region of the CLCuKoV-genome possesses bidirectional promoter</td>
<td>This study</td>
</tr>
<tr>
<td>CLCuKoV-Bu-C1</td>
<td>C1 gene promoter of CLCuKoV-Bu genome</td>
<td>This study</td>
</tr>
<tr>
<td>CLCuKoV-Bu-V1</td>
<td>V1 gene promoter of CLCuKoV-Bu genome</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PIA2</td>
<td>Source plasmid vector containing CaMV35S promoter and Cry1Ac-NOS expression cassette</td>
<td>[30,31]</td>
</tr>
<tr>
<td>pCAMBIA1301</td>
<td>Binary vector used for cloning of gene expression cassettes (promoter +Cry1Ac-NOS)</td>
<td>This study</td>
</tr>
<tr>
<td>PK2-Ac</td>
<td>Positive control plant expression vector containing CaMV35S promoter and Cry1Ac-NOS cassette</td>
<td>[32]</td>
</tr>
<tr>
<td>CLCuKoVC1-Ac</td>
<td>5′-CLCuKoVC1-Cry1Ac-NOS-3′ plasmid</td>
<td>This study</td>
</tr>
<tr>
<td>CLCuKoVV1-Ac</td>
<td>5′-CLCuKoVV1-Cry1Ac-NOS-3′ plasmid</td>
<td>This study</td>
</tr>
<tr>
<td>pBluescript II KS/SK (+)</td>
<td>Cloning vector harboring CLCuKoV-Bu genome</td>
<td>[29]</td>
</tr>
<tr>
<td><strong>Plant Materials</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nicotiana tabacum</td>
<td>Tobacco model plant used for agroinfiltration</td>
<td>This study</td>
</tr>
<tr>
<td>Gossypium hirsutum</td>
<td>Cotton cultivar MNH-786 used for transformation</td>
<td>This study</td>
</tr>
</tbody>
</table>

#### 2.2. Isolation and DNA Sequence Analysis of CLCuKoV-Bu LIR Region

The cloned CLCuKoV-Burewala genome (DNA-A) in this study was previously cloned and sequenced [29]. The CLCuKoV-Bu LIR region was PCR-amplified using the primer P1 5′-GGCGGTACCTGACTTTTGTCGTAATTTRAGACACAC-3′ to obtain the CLCuKoV-Bu fragment comprising nucleotide coordinates 292 to 268, with the addition of KpnI restriction enzyme at the 5′ end, and primer P2 5′-GGCGGTACCTATTCTAGCCCTTTATTACCAG-3′ to obtain the CLCuKoV-Bu fragment comprising nucleotide coordinates from 2595 to 2618, with the addition of KpnI restriction enzyme at the 5′ end. The amplicons harboring the CLCuKoV-BuC1 and CLCuKoV-BuV1 promoters were separated by agarose gel electrophoresis, visualized, eluted and was cloned separately into the Invitrogen TA plasmid vector using PCR 2.1® TA available in the TA cloning kit. To verify the successful TA-cloning of the bidirectional promoter (CLCuKoV-Bu-C1 and CLCuKoV-Bu-V1), the clones were digested using KpnI and visualized by gel electrophoresis, as well as by PCR amplification (Table 2).
Table 2. Oligonucleotide primers, restriction enzymes, and amplicons.

<table>
<thead>
<tr>
<th>Genes/Constructs</th>
<th>Primer Name</th>
<th>Sequence (5’-3’)</th>
<th>Product Size (bp)</th>
<th>Restriction Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cry1Ac-NOS</td>
<td>Cry-NOSF</td>
<td>CGGGTACCATGGGACAAACACCACACACATCAA</td>
<td>2160</td>
<td>KpnI</td>
</tr>
<tr>
<td></td>
<td>Cry-NOSR</td>
<td>CGGGATCCGAATCCCAGTCTAGTAAACATAG</td>
<td></td>
<td>BamHI</td>
</tr>
<tr>
<td>CLCuKoV-Bu-C1</td>
<td>CLCuVC1-F</td>
<td>CGGTTACGCCTTGGTCAATTAGAGACAAC</td>
<td>455</td>
<td>KpnI</td>
</tr>
<tr>
<td></td>
<td>CLCuVC1-R</td>
<td>GGGTACTTTGACCTCTAGGCCITTATTACAG</td>
<td></td>
<td>KpnI</td>
</tr>
<tr>
<td>CLCuKoV-Bu-V1</td>
<td>CLCuVV1-F</td>
<td>CCGGTACGCCTTGGTCAATTAGAGACAAC</td>
<td>455</td>
<td>KpnI</td>
</tr>
<tr>
<td></td>
<td>CLCuVV1-R</td>
<td>CCGGTACCTTGCCTGACGTTTATTACAG</td>
<td></td>
<td>KpnI</td>
</tr>
<tr>
<td>LIR</td>
<td>IR-F</td>
<td>TGACTTTTGGTCAATTAGAGACAAC</td>
<td>455</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>IR-R</td>
<td>TAAATCTAGGCCITTATTACAG</td>
<td></td>
<td>--</td>
</tr>
<tr>
<td>Cry1Ac_internals</td>
<td>Cry-F</td>
<td>TCCCTCTTCCTGCTGCAA</td>
<td>565</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>Cry-R</td>
<td>CGTTCCCATATGTTCCATA</td>
<td></td>
<td>--</td>
</tr>
<tr>
<td>CLCuKoV-BuCl-Cry1Ac-NOS</td>
<td>C1Cry-F</td>
<td>GGATCGGGAATAATGGTCCT</td>
<td>724</td>
<td>--</td>
</tr>
<tr>
<td>orientation</td>
<td>CryC1-R</td>
<td>CGAAGCTTCTCAGTCTCTCG</td>
<td></td>
<td>--</td>
</tr>
<tr>
<td>CLCuKoV-V1-Cry1Ac-NOS</td>
<td>V1Cry-F</td>
<td>CCGTTCACGGGTTCGCAG</td>
<td>228</td>
<td>--</td>
</tr>
<tr>
<td>orientation</td>
<td>CryV1-R</td>
<td>GTGTAACCCGGTTTCAATGCCTT</td>
<td></td>
<td>--</td>
</tr>
</tbody>
</table>

2.3. Construction of Agrobacterium Expression Vectors

The locations of the restriction sites used to clone the Cry1Ac-NOS, CLCuKoV-Bu LIR, and of cloning sites in the pCAMBIA1301 vector was predicted using WebCutter 2.0. The pCAMBIA1301 plant expression vector was used for the agroinfiltration assay in tobacco and also for cotton transformation. Based on the restriction enzyme site in the pCAMBIA1301 vector, the Cry1Ac-NOS cassette of 2160bp was PCR amplified from the source plasmid, PIA-2 (pGEM-Z4-based vector) using sequence-specific primers. Primers were designed based on the full-length Cry1Ac-NOS transcript sequence using the Primer 3 online software version 0.4. Primers were synthesized to obtain the KpnI-BamHI restriction enzymes sites (Table 2). The gel-purified PCR amplicon consisting of the KpnI-BamHI-Cry1Ac-NOS cassette was cloned into the KpnI-BamHI digested pCAMBIA1301 plasmid vector. Both the TA-CLCuKoV-BuCl and TA-CLCuKoV-BuV1 promoter fragments at the 5’ end upstream from the Cry1Ac-NOS were cloned into the pCAMBIA1301 plasmid vector. The orientation and stable integration of the cloned promoters and of Cry1Ac were confirmed by restriction digestion analysis and PCR amplification.

2.4. Transient Expression of Cry1Ac

The plasmid vectors harboring CLCuKoV-BuCl-Ac, CLCuKoV-BuV1-Ac and PK2Ac were transferred into A. tumefaciens strains GV1301 and LBA4404, using the electroporation thaw method and the Bio-Rad electroporation device (cat# 165-2105). The Agrobacterium culture was prepared and used to agroinfiltrate N. tabacum leaves, as previously described [33]. The infiltrated leaf samples were collected at 0, 24, 36, 48, 96 and 120 h post-infiltration. Transient expression of the Cry1Ac gene-encoded protein was confirmed using an immuno strip test (Cat # AS 003 CTLS).

2.5. Agrobacterium-Mediated Transformation in Cotton

The cotton cultivar MNH-786 selected for expression of Cry1Ac is a high yielding genotype approved for routine cultivation in the field, and has been shown to have high regeneration potential [34]. The A. tumefaciens GV1301 harboring CLCuV-Ac (CLCuKoV-BuCl-Cry1Ac-NOS) was used to transform cotton G. hirsutum ‘MNH-786’ using the shoot-apex method [35]. This transformation procedure has been previously optimized at the CEMB Laboratory. Briefly, cotton seeds were acid de-linted, surface sterilized in Tween-20...
for 3 min, and transformed to a solution containing 0.1% HgCl₂ and Sodium dodecyl sulphate (SDS). The sterilized seeds were held at 30 °C for 1–2 days in the dark. The shoot-tips of the germinating embryos were excised using a sharp sterilized blade and immediately transferred to the A. tumefaciens inoculum suspension carrying CLCuV-Ac and PK2-Ac plasmids. These embryos were transferred to Murashige Skoog MS-medium (kinetin-1 mg/mL) antibiotic-free media for 3 days at 25 °C. After three days of co-cultivation, the cotton plantlets were transferred to a sterile test tube containing MS with hygromycin (50 µg/mL) and cefotaxime (250 µg/mL). When desirable, higher hygromycin concentrations were used to reduce the rate of false-positive transformants (data not shown). A total of 5000 cotton embryos were isolated during the plant transformation experiment (Table 3).

Table 3. Transformation efficiency of constructs in cotton variety MNH-786.

<table>
<thead>
<tr>
<th>Constructs</th>
<th>No. of Embryos</th>
<th>Survival % (3–4 Week)</th>
<th>Survival % (7–8 Week)</th>
<th>Plants in Soil</th>
<th>Transformation Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLCuV-Ac</td>
<td>3000</td>
<td>375</td>
<td>190</td>
<td>14</td>
<td>0.46</td>
</tr>
<tr>
<td>PK2-Ac</td>
<td>2000</td>
<td>125</td>
<td>70</td>
<td>7</td>
<td>0.35</td>
</tr>
</tbody>
</table>

2.6. Molecular Analysis of Transgenic Cotton Plants

Genomic DNA was extracted and purified from new growth (terminal) leaves of cotton plants selected for positive transformation using a modified (combination of two) DNA isolation method [36,37]. The detection of the internal fragment of the Cry1Ac gene was carried out by PCR amplification with the Cry1Ac specific internal primer set (Table 2). The cycling conditions were 95 °C for 3 min followed by 30 cycles 95 °C (30 s), 54 °C (30 s) and 72 °C (40 s), with final extension of 7 min (72 °C). A commercially-available ELISA assay was used to evaluate the relative expression of Bt-Cry1Ac toxin in newly transformed cotton plants using Envirologix Kit (Cat # AP051).

2.7. Statistical Analysis

The Cry1Ac quantification expression was analyzed statistically in completely randomised designs (CRD). One-way ANOVA followed by Tukey’s HSD analysis was performed using Statistix 8.1 software (Statistix 8.1, Tallahassee, FL, USA).

3. Results

3.1. Construction of Plasmids Containing Single (Cry1Ac) Gene Constructs

The presence of the Cry1Ac-NOS gene, CLCuKoV-BuC1 and CLCuKoV-BuV1 promoter in the pCAMBIA-1301 were confirmed by PCR using Cry1Ac internal primers and primers designed to amplify the specified orientation of the cloned inserts (Table 1) and by restriction digestion, as described. Ultimately, two plasmids were obtained successfully by cloning the Cry1Ac-NOS gene cassette in the binary pCAMBIA1301 vector. The final gene constructs were referred to as CLCuKoV-BuC1-Ac consisting of 5′-CLCuKoV-BuC1-Cry1Ac-NOS-3′ and CLCuKoV-BuV1-Ac consisting of 5′-CLCuKoV-BuV1-Cry1Ac-NOS-3′ as illustrated in (Figure 1). Both single gene constructs were shown to be transferred into A. tumefaciens GV1301 competent cells, used for Agrobacterium-mediated transient plant transformation, and stable transformation in cotton was obtained. The PK2-Ac consisting of a CaMV35S-Cry1Ac-NOS cassette (2476 bp) harboring the CaMV35S promoter that is most commonly used for plant transformation, was also stably transformed into cotton embryos.
Figure 1. (A) Diagram showing the genome organization of CLCuKoV-Bu. The five begomoviral ORFs are indicated by colored arrows. The viral plus strand encodes the V1 and V2 ORFs while the complementary strand encodes three ORFs, C1, C3 and C4. The viral coding regions are separated by a non-coding large intergenic region (LIR) of 455 nucleotides in size. (B) Diagram of the LIR shows the predicted regulatory motifs, cis-regulatory elements, transcription factor binding sites, and iteron elements involved in binding the replication-associated protein. (C) Map of the Cry1Ac gene expression cassettes under control of the CLCuKoV-BuC1 and CLCuKoV-BuV1 promoters. The Cry1Ac+ NOS was cloned at the restrict site Kpn1-BamH1 in the pCAMBIA1301 plasmid vector.
3.2. Transient Expression of Cry1Ac Gene

Both bidirectional promoter gene constructs, CLCuKoV-BuC1-Ac and CLCuKoV-BuV1-Ac, were shown to be expressed transiently in *N. tabacum* using an *Agrobacterium*-mediated transient expression assay. Three days post-agroinfiltration, the Cry1Ac protein was detected using the commercially-available immunostrip assay, indicating robust transient expression of the Cry1Ac gene in infiltrated tobacco leaves when driven by begomoviral promoters. No protein expression was observed in the negative control tobacco leaves agroinfiltrated with CLCuKoV-BuC1-Ac and CLCuKoV-BuV1-Ac, alone, as expected (Figure 2).

![Image of immunostrip assay results](image-url)

**Figure 2.** Immunoassay results of transient expression of the Cry1Ac gene in *N. tabacum* leaves transiently transformed with the Cry1Ac constructs. Lane 1 contains extracts of cotton plants infiltrated with the construct driven by the CLCuKoV-BuC1 promoter in which Cry1Ac protein was detected. Lane 2 contains extracts of cotton plants infiltrated with CLCuKoV-BuC1 minus the bidirectional promoter, in which no Cry1Ac protein was detectable.

3.3. Transformation of Single Gene Construct in *G. hirsutum*

The CLCuV-Ac construct harboring the begomoviral bidirectional promoter of the begomoviral AC1 gene was selected for transformation experiments (hereafter, CLCuV-Ac). Germinating embryos of cotton ‘MNH-786’ were transformed with the CLCuV-Ac and PK2-Ac cassettes, using the *Agrobacterium*-mediated shoot-apex method. Three thousand cotton embryos were used for transformation experiments for the CLCuV-Ac construct, and 2000 embryos were used for transformation experiments for PK2-Ac construct. The putatively transformed cotton plants were screened by antibiotic selection medium containing 75 mg/mL of hygromycin.

After two months of selection, one-hundred and ninety, and seventy plantlets putatively transformed with the CLCuV-Ac and PK2-Ac constructs, respectively were recovered (Table 3). Fourteen and seven plants presumed to be transformed with the CLCuV-Ac and PK2-Ac constructs, respectively, were grown transferred to pots containing soil and maintained in the CEMB-Greenhouse. Among these plants, five were phenotypically abnormal. Transformation efficiency was 0.46% and 0.35% for the CLCuV-Ac and PK2-Ac construct, respectively (Table 3). After 1–2 weeks, total DNA was isolated from the plants and tested...
for presence of the respective constructs by PCR amplification using construct-specific primers, as described. The results indicated two plants each harboring the CaMV35S and the bidirectional begomoviral promoter cassettes, respectively, were PCR-positive for transformation (Figure 3). The transformed cotton plants were maintained in the CEMB greenhouse and subjected to molecular analysis.

**Figure 3.** Detection of the Cry1Ac gene fragment in transformed cotton plants by PCR amplification. Lanes 2–9 showed the amplicons obtained for the Cry1Ac gene (565 bp) in putatively transformed cotton plants. Lane 1 contains the 1 Kb p DNA marker. Lanes 2–5 contains amplicons from the CLCuV-Ac transformed cotton plants. Lanes 6–9 contains amplicons from PK2-Ac transformed cotton plants. Lane 10 is the negative control, and Lane 11 contains the amplicons from the plasmid CLCuV-Ac used as the positive control.

### 3.4. Molecular Analysis of Putative Cotton Plants

The presence of the Cry1Ac gene integrated into the genome of newly transformed cotton plants were verified by PCR amplification for the CLCuV-Ac and PK2-Ac transgene, respectively (Figure 3). To evaluate Cry1Ac endotoxin level driven by two different promoters, the relative accumulation of Cry1Ac endotoxin protein expression driven by CaMV35S (PK2-Ac) and the bidirectional begomoviral promoter (CLCuV-Ac), respectively, were monitored at 15-day intervals for 8 weeks. Consistent expression of the endotoxin Cry1Ac gene driven by the CLCuKoV-BuC1 promoter (CLCuV-Ac) was observed for the two transgenic cotton plants obtained here. The Cry1Ac protein was detected by ELISA in transformed cotton plants expressing the Cry1Ac gene driven by the CaMV35S promoter, however, relative endotoxin accumulation declined over time, compared to plants expressing the transgene driven by the begomoviral bidirectional C1 promoter (Figure 4). The maximum Cry1Ac protein was detected in leaves, followed by bolls, squares, and then the anthers. All the recorded differences were highly significant as revealed by a one-way ANOVA followed by Tukey’s HSD analysis. The statistical analysis revealed that there were significant differences ($p < 0.05$) for all cotton plants (Figure 4). The lowest coefficient of variance was observed for PK2-Ac 90-day-old plants, while it was higher for CLCuV-Ac plants (Figure 4). No Cry1Ac endotoxin was detected in the roots of transgenic cotton plants (Figure 5).
4. Discussion

During the last two decades, commercial transgenic Bt cotton plants have been developed for insect pest control. These plants have descended from a common parent, “Monsanto-531”, which was obtained by agro-transformation. The latter plants harbor the expression cassette containing the Cry1Ac gene driven by the CaMV35S promoter. The CaMV35S drives the expression of Cry1Ac protein in nearly all parts of the cotton plant, and it is by far the most widely-used promoter for plant transformation, especially for dicotyledonous species, including cotton [38–40]. Although the CaMV35S promoter has been highly useful for driving constitutive gene expression in transgenic plants, several studies have shown that expression levels may vary throughout the growing season, possibly due to interactions associated with particular physiological stages of plant growth and/or environmental factors [41,42]. There are no available means of regulating candidate gene expression temporally by growth stage or spatially, in plant parts, given that cellular energy is required for all processes but cannot be harnessed and modulated [10,43]. Thus,
identifying alternative promoters to drive gene expression in green tissues of plants has become increasingly desirable.

Agrobacterium-mediated transient transformation offers a powerful tool for rapid evaluation of transgenes [33,44,45], promoters [19], and gene editing in crop plants [46]. In a previous study, the LIR of the CLCuKoV-Bu was characterized and shown to function as an effective bidirectional promoter in agroinfiltration assays using GUS as the reporter gene [19]. In this study, the LIR was subcloned from a previously available CLCuKoV-Bu genomic clone [29] and the bidirectional promoter activity to drive Cry1Ac gene expression was verified using a transient assay, prior to stably transforming cotton plants with the promoter driving expression of the Cry1Ac gene. The purpose of this study was to construct a cassette with a single gene, Cry1Ac, and demonstrate the potential effectiveness of two begomoviral bidirectional promoters to drive robust Cry1Ac expression transiently, and/or in transgenic cotton, compared to the CaMV35S promoter. To achieve robust expression in cotton, the binary vector pCAMBIA-1301, previously used to achieve high levels of gene expression in cotton, was selected as the plasmid vector for expressing Cry1Ac driven by the bidirectional CLCuKoV-BuC1 and CLCuKoV-BuV1 promoters by Agrobacterium-mediated transformation (Figure 1).

The Cry1Ac transient expression assays were carried out 72 h post-infiltration using a commercially-available immunostrip assay. No Cry1Ac protein was observed in the CLCuKoV-BuV1-Ac agroinfiltrated leaves (Figure 2), a result that was inconsistent with a previous report. These results observed in this study system indicate that CLCuKoV-BuV1 is a weak promoter, or unable to drive Cry1Ac expression in the agroinfiltrated leaves, and support those reported from a previous study that evaluated the robustness of the CLCuKoV-Bu bidirectional promoter [19].

PCR detection of insect resistant genes and event in transgenic cotton is most widely used an efficient and highly reliable method. It is based on the stable nature of the genomic DNA and sensitivity of the PCR methodology [47,48]. PCR amplification from transgenic cotton plants using gene-specific internal primers of Cry1Ac-CEMB has validated the results. A prerequisite for a successful regulatory framework and commercial approval process is the biosafety evaluation of transgenic events, with molecular characterization as an important component [49]. The expression of Cry1Ac gene among cotton cultivars can vary throughout the growing season in Pakistan [8].

PCR analyses of transgenic cotton plants confirmed the amplification of a 565 bp product corresponding to Cry1Ac gene (Patent No. K140649-aroA) verifying the presence of Cry1Ac gene in the genome of cotton test plants (Figure 3). One month-old PCR positive-cotton plants were assessed relative accumulation of the CEMB-Cry1Ac toxin using the ELISA assay. By 90 days post-transformation, Cry1Ac toxin accumulation was three-fold higher in the cotton leaves expressing the transgene was driven by the CLCuKoV-BuC1 promoter, compared to CaMV35S driven expression in cotton plants (Figure 4). This is consistent with the results of a previous study, in which the CaMV35S promoter showed low Cry1Ac toxin levels in transgenic cotton leaves [10].

Commercialization of Bt toxin-expressing cotton plants under control of the CaMV35S promoter has been of great interest for protecting the cotton crop against lepidopterans insect pests in Pakistan. A number of insect pests have developed resistance against the Bt toxin, resulting from naturally-occurring evolutionary processes that lead to selection. Mutations in the cadherin and ABC transporter genes have been identified as target sites responsible for the development of insect resistance to Cry1Ac toxin [50]. To enhance the accumulation of the Cry1Ac toxin in cotton leaves, different promoters for driving gene expression were evaluated. In a previous study, an rbcS promoter has been shown to enhance expression of the Cry1Ac gene in cotton [10]. Similarly, a maize Ubiquitin promoter has been used to enhance expression of Cry1Ac in transgenic sugarcane [26], while the PNZIP (Pharbitis nil leucine zipper) promoter was shown to enhance Cry9C expression in cotton. However, expression of the CaMV35S promoter has been shown to be
inconsistent and/or to express lower than desirable levels of Cry9C in Bt transgenic cotton at certain times during the growing season [51].

In this study, the use of a begomoviral bidirectional promoter to enhance expression of the Cry1Ac gene was explored for the first time in a field-ready cotton cultivar. The CLCuKoV-BuC1 promoter was shown to drive Bt toxin expression in green tissues of cotton, including in leaves, bolls, squares, and anthers, while at the same time Bt protein was minimally detectable in cotton roots (Figure 5), and represents a feature that may aid in alleviating public concerns about the environmental safety of transgenic crop protection strategies. The results of this study demonstrate that the CLCuKoV-BuC1 promoter is capable of robust expression of the Cry1Ac gene and its accumulation in leaves and bolls of cotton plants for at least eight weeks post-transformation. The observation may pave the way to an understanding of plant physiology and development, particularly with respect to the mechanisms involved in modulating spatiotemporal variations in transgene expression, not only of Cry1Ac, but of other transgenes, as well, during different plant growth stages.


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**References**


9. Adamczyk, J.J.; Perera, O.; Meredith, W.R. Production of mRNA from the Cry1Ac transgene differs among Bollgard® lines which correlates to the level of subsequent protein. *Transgenic Res.* 2009, 18, 143–149. [CrossRef]


40. Bak, A.; Emerson, J.B. Cauliflower mosaic virus (CaMV) biology, management, and relevance to GM plant detection for sustainable organic agriculture. *Front. Sustain. Food Syst.* 2020, 4, 21. [CrossRef]