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Short Communication

Reconstruction and validation of three different binary vectors suitable for generation of genetically engineered *Helicoverpa* protected crops

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Abstract

Availability of a suitable plant transformation binary vector is necessary for the generation of transgenic crops with an adequate expression of transgenic proteins. Therefore, three binary vectors were constructed viz., pBK204, pBK205, and pBK206 harboring either a truncated or a full-length version of a *Cry1Ac* gene for the generation of *Helicoverpa* protected crops. Two different promoters viz., Arabidopsis Rubisco small subunit (AtSSU) gene promoter or CaMV35S promoters were used to regulate the various versions of *Cry1Ac* gene. The binary vectors were reconstructed either by the Gibson assembly method and others by ligating the restriction enzyme digested fragments. The reconstructed binary vectors were mobilized into *Agrobacterium strain* AGL1 and validated by *Agrobacterium* infiltration assays of *Nicotiana benthamiana*. The amount of Cry1Ac protein accumulated in the Agroinfiltrated tobacco leaves was quantified using the quantitative ELISA assay. The expression of the Cry1Ac protein in the tobacco leaves ranged from 0.25 to 0.26 µg /g fresh weight (FW) when transformed with these three constructs. Thus, the vectors constructed in this study appeared to be suitable for generation of *Helicoverpa* resistant transgenic crops by *Agrobacterium*-mediated genetic transformation method.

Keywords: Binary vector, *Cry1Ac*, agroinfiltration, transformation

Construction of a suitable binary vector is always important for the *Agrobacterium*-mediated genetic transformation of crops. pBin19 is the first reported binary vector (Bevan 1984) followed by pART (Gleave et al. 1992) and pPLEX501 Schunmann et al. (2003). *Cry1Ac* gene isolated from *Bacillus thuringiensis* gene is one of the important insecticidal genes to protect *Helicoverpa*. Both truncated and the full-length *Cry1Ac* gene is reported to provide a considerable level of protection against Lepidopteran insects in transgenic cotton (Perlak et al. 1990; Perlak et al. 2001; Purcell et al. 2004) and transgenic tomato (Koul et al. 2015). Suitable promoters are essential to regulate the gene of interest for the optimal level of expression of transgene protein. Cauliflower mosaic virus (CaMV) is one of the broadly used constitutive promoters in transgenic plants (Odell et al. 1985). Tissue-specific *Arabidopsis* rubisco small subunit (AtSSU) gene promoter was also reported to induce a higher level of expression of the Cry1Ac protein in tobacco plant when compared with the levels of Cry1Ac protein regulated by the CAMV35S promoter (Wong et al. 1992). Later, the AtSSU gene promoter is also reported to accumulate high levels of *Cry1Ac* (*Tic107*) gene in soybean chloroplasts (Miklos et al. 2007) and chickpea (Acharjee et al. 2010). Apart from the gene promoter combination, the efficiency of the plant transformation vector relies on the efficient recovery of transformed cells using suitably selectable marker gene. *Neomycin phosphotransferase* II gene is one of the comprehensively used selectable marker genes in transgenic plant (Brian et al. 2004). Once the

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combination of the above features is achieved in a binary vector, it is essential to find out whether the reconstructed vector would be useful for crop improvement or not. Transient expression assay that facilitates easy monitoring of the recombinant protein production is efficiently used for this purpose. In this present study, an attempt was made to reconstruct three plant transformation vectors harboring either truncated or full-length *Cry1Ac* gene regulated either by the CaMV35S promoter or AtSSU gene promoter. A transient expression assay was carried out to validate the efficiency of the constructs. There are no previous reports on the use of *Cry1Ac* full gene driven by AtSSU gene promoter for the generation of transgenic plants.

Sticky end cloning method was used to construct binary vector pBK204 harboring a full-length *Cry1Ac* gene regulated by AtSSU promoter as illustrated in (Fig. 1). Transformed DH5α cells harboring the pBK204 were confirmed by restriction digestion using BamHI, PvuII and HindIII restriction enzymes (Fig. 2). Sequencing revealed the presence of a full-length *Cry1Ac* sequence of 3.5 kb size in pBK204, similar to the sequence described by Adang et al. (1985). Another two binary vectors *viz*., pBK205 harboring truncated *Cry1Ac* gene and pBK206 harboring full-length *Cry1Ac* gene regulated by the CaMV 35S promoter were reconstructed following the Gibson assembly cloning method as illustrated in Figs. 3 and 5. Sequence-specific primers were used to amplify the fragments of pBK205 and pBK206, respectively (Supplementary Tables S1 and S2, www.isgpb.org) so that the amplified products contain at least 18 to 20bp overlapping regions between the adjacent DNA fragments. Amplified fragments are further ligated using the Gibson assembly master mixture followed by transforming DH5ά competent cells. Transformed DH5ά cells were confirmed by restriction digestion using SalI restriction enzyme for pBK205, (Fig. 4) and (BamHI and SalI) for pBK206, (Fig. 6). The confirmed recombinant clone was mobilized into *Agrobacterium strain* AGLI for transient expression assay. Syringe infiltration method of *Agrobacterium* culture was used for transient expression assay for the validation of recombinant constructs. Tobacco (*Nicotiana benthamiana* L.) was used as a model plant for this purpose. We followed the protocol described by Sparkes et al. (2006) and used *Agrobacterium* strain AGL1 harboring plasmids pBK203 or pBK204 or pBK205 or pBK206 and a viral suppressor of PTGS plasmid, p19, to alleviate the host silencing in wild-type tobacco.

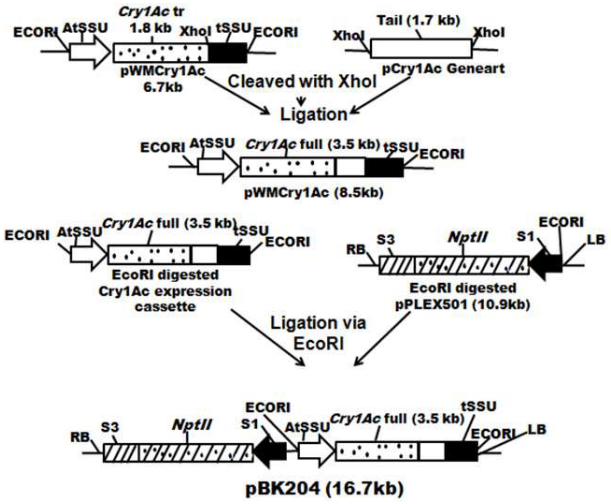

Fig. 1. Strategy for construction of recombinant vector pBK204. AtSSU, Arabidopsis rubisco small subunit promoter depicted in white arrow; *Cry1Ac* tr, *Cry1Ac* truncated gene from *Bacillus thuriengensis* depicted in white block with black dots; tSSU, Small subunit terminator from tobacco is depicted in black block; Tail, 3' end portion of full length *Cry1Ac* gene (1.7kb) depicted in white block; *Cry1Ac* full, Full length *Cry1Ac* gene of size 3.5 kb after joining of 3'end portion to the truncated *Cry1Ac* region depicted in white block with black dots and white region; S1, Promoter from Subclover stunt virus depicted in black arrow; *NptII*, Neomycin phosphotransferase (kanamycin resistance) depicted in block with stripes and dots; S3, Terminator from Subclover stunt virus depicted in box with stripes; LB and RB, Left and Right borders of T-DNA region; XhoI and EcoRI, Restriction sites

Transient expression of Cry1Ac protein in Nicotiana benthamiana

ELISA assay was performed to determine the transient expression of Cry1Ac protein in tobacco leaves infiltrated with reconstructed vector pBK204 or pBK205 or pBK206 after three days of incubation. The procedure was followed as per the *Cry1Ac/Cry1Ab* ELISA assay kit of Agdia (Catalog No. PSP06200). Three replicas of plant sample were utilized to carry out the ELISA test for each construct. Quantitative ELISA of tobacco leaves transformed with pBK204, pBK205 and pBK206 confirmed transient expression of Cry1Ac protein ranging from 0.25 to 0.26 µg/g FW when co-infiltrated with viral suppressor of PTGS plasmid, p19 (Fig. 7), (Supplementary Table S3). Transient expression of Cry1Ac protein at the level of 0.27 µg/g FW was also observed in tobacco leaves

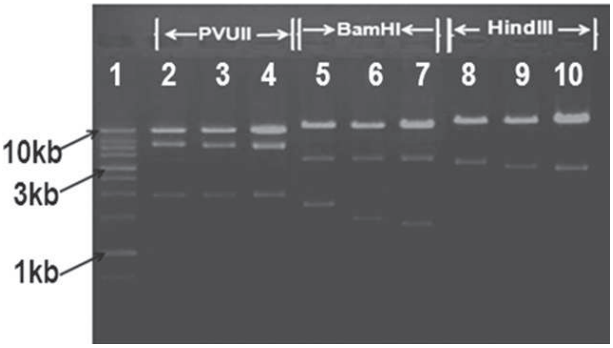

Fig. 2. Restriction mapping of pBK204, Lane 1: Ladder, Lane 2 to 4: Digestion with PvuII, Lane 5 to 7: Digestion with BamH1, Lane 8 to10: Digestion with HindIII. Lanes: 2, 5, 8 (represent single colony showing expected bands of size (9.2 kb, 5.5 kb, 2 kb) for PvuII, (11.3 kb, 3.6 kb, 1.7kb) for BamHI and (13.3kb, 3.3kb) for HindIII respectively

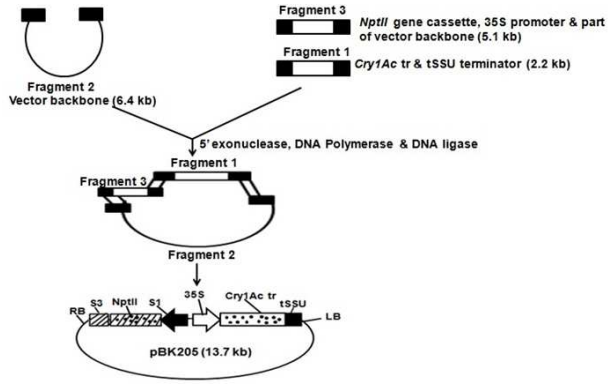

Fig. 3. Strategy for construction of recombinant vector pBK205. Fragment 1, contain the *Cry1Ac* truncated gene and small subunit terminator from tobacco; Fragment 2, contain the vector backbone; Fragment 3, contain part of vector backbone, *NptII* gene cassette and 35S promoter; Black blocks depicts the overlapping region between the adjacent sequences; 35S, 35 promoter from Cauliflower Mosaic Virus depicted in white arrow; *Cry1Ac* tr, *Cry1Ac* truncated gene from *Bacillus thuriengensis* depicted in white block with black dots; tSSU, Small subunit terminator from tobacco is depicted in black short block; S1, Promoter from Subclover stunt virus depicted in black arrow; *NptII*, Neomycin phosphotransferase (kanamycin resistance) depicted in block with stripes and dots; S3, Terminator from Subclover stunt virus depicted in block with stripes; LB and RB, Left and Right borders of T-DNA region

when p19 was co-infiltrated with a control construct pBK203 harboring a truncated *Cry1Ac* gene regulated

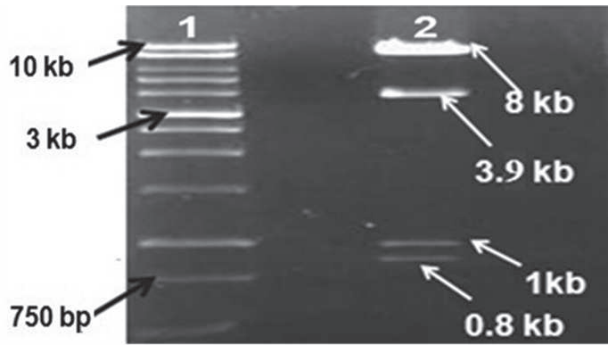

Fig.4. Restriction mapping of pBK205 with SalI, Lane1: Ladder, Lane2: Digestion of pBK205 with SalI with expected bands of size (0.8 kb, 1.0 kb, 3.9 kb and 8.0 kb)

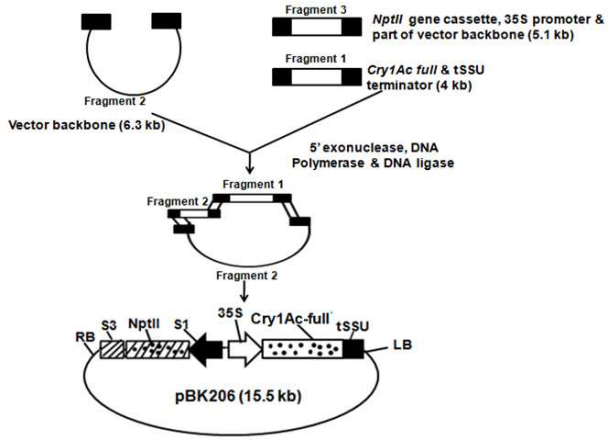

Fig. 5. Strategy for construction of recombinant vector pBK206. Fragment 1, contain the *Cry1Ac* full gene and small subunit terminator from tobacco; Fragment 2, contain the vector backbone; Fragment 3, contain part of vector backbone, NptII gene cassette and 35S promoter; Black block depicts the overlapping region between the adjacent sequences; 35S, 35 promoter from Cauliflower Mosaic Virus depicted in white arrow; *Cry1Ac* full, *Cry1Ac* full gene from *Bacillus thuriengensis* depicted in white block with black dots; tSSU, Small subunit terminator from tobacco is depicted in black short block; S1, Promoter from Subclover stunt virus depicted in black arrow; *NptII*, Neomycin phosphotransferase (kanamycin resistance) depicted in block with stripes and dots; S3, Terminator from Subclover stunt virus depicted in block with stripes; LB and RB, Left and Right borders of T-DNA region

by AtSSU promoter. The vector pBK203 has been proved to generate stable transgenic chickpea plants with high-level expression of Cry1Ac protein (>79.9 ng1Ac/mg FW) by our group (Acharjee et al.

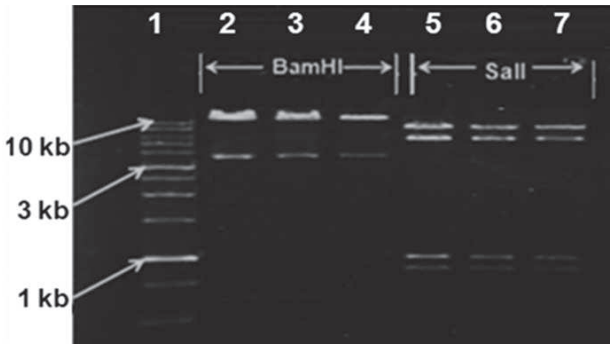

Fig. 6. Restriction mapping of pBK206 with BamHI and SalI, Lane1: Ladder, Lane2 to 4: Digestion with BamHI with expected bands of size (0.6 kb, 3.5 kb, & 11.3 kb), Lane 5 to 7: Digestion with SalI with expected bands of size (0.8 kb, 1kb, 5.6kb & 8kb)

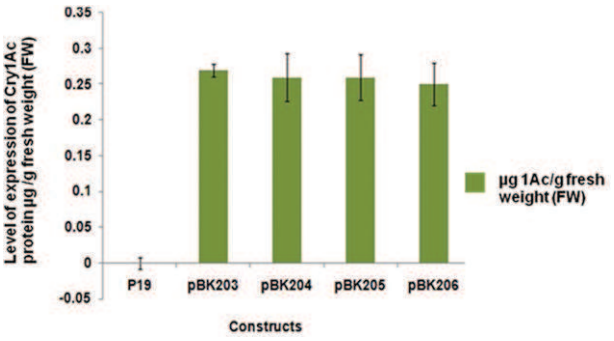

Fig. 7. Transient expression of Cry1Ac protein in tobacco leaves infiltrated with *Agrobacterium* harbouring recombinant constructs pBK204, pBK205, pBK206. pBK203: Positive control, p19: Viral RNA silencing suppressor protein

unpublished). The standard deviation of transient protein level from the three replicas was calculated for each construct which was found to be less than 13% of the mean value. Unpaired t-test data revealed no significant variation in transient expression efficiency between the control construct (pBK203) and the tested constructs pBK204, pBK205 and pBK206 (t-test, $p>0.05$), (Supplementary Table S3).

Transient expression system via Agroinfiltration thus revealed the suitability of the reconstructed binary vectors for transient Cry1Ac protein production. The advantage of the transient expression system is unlike stable transformation; the method enables quick monitoring of the transgene protein only after 2 to 3 days of infiltration. There are reports of the use of this method for many purposes including foreign gene expression (Kapila et al. 1997), promoter and transcription factor validation (Yang et al. 2000), recombinant protein production (Jin et al. 2015). Validation of numbers of viral and nonviral vectors constructs was also reported by using a transient expression system *via* Agroinfiltration (Shah et al. 2013). The group suggested that often non-viral vectors (pPZP5025 and pPZP3425) are needed to be co-infiltrated along with the RNA-silencing inhibitor p19 for appropriate levels of expression of reporter genes, while viral vector such pPZPTRBO, pJLTRBO, and pEAQ-HT showed similar expression levels without co-infiltration of an RNA-silencing inhibitor. It was reported that in *N. benthamiana* suppression of post-transcriptional gene silencing (PTGS) was achieved by expression of the tomato bushy stunt virus (TBSV) p19 protein (Voinnet et al. 1999). Thus, co-infiltration of the p19 vector along with plant transformation vector is also known to facilitate higher accumulation transgenic protein with a short period.

In the present study, these reconstructed pPLEX501 based binary vectors unlike pBIN19 contain *nptII* gene under the control of a strong constitutive S1 promoter derived from Sub Clover Stunt Virus (SCSV) genome. So far, the CaMV35S promoter has been used predominantly as a strong promoter for efficient selection of transgenic plants. There are reports that frequent use of the similar promoter may lead to transgene silencing (Matzke et al. 1994; Park et al. 1996). Other strong promoters of plant housekeeping gene actin, maize alcohol 12 dehydrogenase1 (*Adh1*) and ubiquitin have been utilized in the transgenic plants, but mainly in monocots (reviewed in Shah et al. 2015) and so far not in any commercially available product of agricultural biotechnology. The efficient recovery of high expressing transgenic chickpea plants has been reported from our group using S1 promoter driven *npt*II gene (Acharjee et al. 2010). Moreover, unlike the pBin19, pPLEX501 contained a high-copy-number origin of replication for *E. coli* which facilitates easy cloning.

In conclusion, this study reports construction of a set of novel binary vectors and its application in generation of stable transgenic lines. The transient expression assay revealed equal efficiency of both CaMV 35S promoter and AtSSU promoter in regulating transient Cry1Ac protein production in *N. benthamiana*.

Authors' contribution

Conceptualization of research (SA, BKS); Designing of the experiments (SA, BKS); Contribution of

experimental materials (BKS); Execution of field/lab experiments and data collection (NH, RRB); Analysis of data and interpretation (NH, SA, BKS); Preparation of manuscript (NH, SA, BKS, PJH).

Declaration

The authors declare no conflict of interest.

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Suplementary Table S1. Primer list to clone pBK205

Brick name	Primer	Primer sequences	Tm
Fragment 1	Forward	5'attgaaaccggttacactccCATCGACATCTCCTTGTCCTTGACACAG-3'	61.4^{0}C
	Reverse	5'gcggacgtttttaatgtactGAATTCGAGCTCGGTACCCTCGAC-3'	60.8^{0}C
Fragment 2	Forward	5'agggtaccgagctcgaattcAGTACATTAAAAACGTCCGCAATGTGTTATTAAG TTGT-3'	60.2^{0}C
	Reverse	5'gcgatcaccgcttccctcatGATGTTTAACTCCTGAATTAAGCCGCGCC-3'	61.5^{0}C
Fragment 3	Forward	5'taattcaggagttaaacatcATGAGGGAAGCGGTGATCGCCGAA-3'	60.8^{0}C
	Reverse	5'aggacaaggagatgtcgatgGGAGTGTAACCGGTTTCAATGCGTTCTC-3'	61.4 ^{0}C

Suplementary Table S2. Primer list to clone pBK206

Brick Name	Primer	Primer sequences	Tm
Fragment 1	Forward	5'attgaaaccggttacactccCATCGACATCTCCTTGTCCTTGACACAG-3'	61.4^{0}C
	Reverse	5'gggagctgttggctggctggTGGCAGGATATATTGTGGTGTAAACAAATTGACG-3'	60.8^{0}C
Fragment 2	Forward	5'caccacaatatatcctgccaCCAGCCAGCCAACAGCTCCC-3'	60.0^{0}C
	Reverse	5'gcgatcaccgcttccctcatGATGTTTAACTCCTGAATTAAGCCGCGCC-3'	61.5^{0}C
Fragment 3	Forward	5'taattcaggagttaaacatcATGAGGGAAGCGGTGATCGCCGAA-3'	60.8^{0}C
	Reverse	5'aggacaaggagatgtcgatgGGAGTGTAACCGGTTTCAATGCGTTCTC-3'	61.4^{0}C

Suplementary Table S3. Expression level of Cry1Ac protein in tobacco leaves infiltrated with construct pBK203 or pBK204 or pBK205 or pBK206 when co infiltrated with p19

Constructs	Expression level of Cry1Ac (µg/g FW protein), Mean ±SD	t-test value *	probability
p19	0.01±0.008	0.00	P<0.05***
pBK203	0.27±0.009	-	
pBK204	0.26±0.033	0.46	P>0.05**
pBK205	0.26±0.032	0.68	P>0.05**
pBK206	0.25±0.029	0.34	P>0.05**

*T-test value was calculated when the expression level induced by tested construct was compared with the control construct pBK203
** Expression level induced by tested constructs were not significantly different when compared to control construct pBK203
*** Expression level induced by p19 is significantly different when compared to control construct pBK203