

Construction and Development of pSB111 Bar Plasmid Vector for Activation Tagging

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Abstract

To get desired traits and to know the precise expression patterns of genes in the plant, *Agrobacterium tumefaciens* has been widely employed in the generation of transgenic plants using plasmid vectors. Many methods are come forward to establish the gene expression of unknown genes but in these techniques gene alteration is the main drawback or sometimes it is lethal to organisms also, to overcome these problems activator tag method developed to know the specific expression of genes at different stages of growth by activating the genes of an organism. A reporter gene with a modest promoter is included in the activator tag vector. The basic principle behind is the expression of reporter gene of activator vector by elucidating the tagged gene expression. Using the Trans configuration of vir genes from the plasmid *Agrobacterium tumefaciens* to transfer right and left sequence bordered T-DNA into the nuclear genome of plants, we designed a vector molecule that promotes expression of a specific gene at more than four times its normal expression and is useful for efficient transformation to higher plants. In this study we modified activator vector by inserting the 4x activator which shows four times effectiveness than normal activator to get the more promising results. To tag and know the genes and their expression profiles, we produced a binary vector consisting of 1.8 kb GFP cassette as a reporter gene and 1.4 kb tetramer of CaMV35S activator (4X-Ac) cloned at HindIII site of pSB11 bar intermediate vector. The recombinant clone harbouring different expressions units were mobilized into *Agrobacterium tumefaciens* through DH5α cells triparental mating to produce a super binary vector pSB111-bar-4xAc-GFP. The generated vector is beneficial to produce transgenic lines of different plant species.

Keywords: pSB11 bar vector, *Agrobacterium tumefaciens*, Activator tagging, CaMV35S activator, GFPcassette.

INTRODUCTION

The advancement of genetic engineering application leads to development of many vectors for transferring foreign genes to host produce transgenic lines in various plants. The vector is a basic tool

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for transformation of specific genes into the nuclear genome of organisms. Plasmids are specially designed to serve the generating transgenic plants. In mid-1980s emerged the frameworks of current development of vector construction for transformation of higher plants. Farley et al., reported that crown gall tumour genesis favours the genetic transformation of plant cells. Hoekema et al., [1] achieved the removal of tumour causing genes from Ti plasmid of *Agrobacterium* LBA4404 and produce disarmed strain. For genes transformation into the plants the *Agrobacterium* has been widely used because of its nature to cause infection by transferring the vir genes into the genome of plants, hence it is called as natural genetic engineer for plants. It is capable of causing

infectious to approximately 140 dicot species [2]. The binary vector is composed of LB and RB, Multiple cloning sites, Ori for *E.Coli* and *Agrobacterium*, reporter genes, selectable markers, desired genes and virulence genes which promotes high frequency of transformation. By the advancement of genome sequence projects many genes were discovered without knowing their proper functions. Many methods are come forward to describe the gene function or identity of genes, among gene tagging using an activator has an advance approach to know the functions of specific genes. They are different types of gene tag such as activator tag, enhancer trap, promoter trap, Poly A trap and gene trap for obtaining conventional mediated insertional mutagenesis in the genome of organism. These systems successfully in identifying the genes in *Drosophila*, Mouse and yeast [3]. Here we focused to develop a vector which worked on the principle based on Activator tagging. The activator tagged vector consists of a reporter gene with minimal promoter, TATA box and transcriptional start site which can be activated by neighbouring activator elements. This construct comprises a visible marker gene downstream of a minimal promoter. The reporter gene in activator tag expressed only when the vector construct is integrated with endogenous activator of host. Hence, in the process of vector construction we slightly modify the activator tag by inserting the 4X activator which is mostly used in activation tagging techniques. Activation tagging is well known approach to reveal the function of genes at different stages of growth by generating randomly insertional mutagenesis [4–7]. Insertion mutagenesis is an effective strategy for mutagenesis and cloning of genes responsible for mutant phenotypes [8–10]. Activation tagging constitutes repeated tandem copies of activator for enhancing the transcription of neighbour flanking genes expression by inserting T-DNA fragment randomly into the plant contains 50–1500 bp bound by transcriptional activator proteins. The main motto behind inserting 4X activator is can activate genes 7 kb away from the inserting site in the genome. It is in Cis configuration mostly sometimes it also found in Trans configuration to transcribe the genes away from 1mbp. CaMV widely used as an activator for plants genetic engineering to transcribe the genes. CaMV is an infectious agent in plants mainly for crucifers [11, 12] proved that CaMV has a hotspot genes for their expression in transgenic plants it have a several domains with different specificity for specific tissues in plants. In the agronomic application 35S used widely in enhancing the expression of heterologous genes in genetically modified plants [13, 14] developed an activation vector of pER38 which contains double tandem CaMv35S activator and transformed into Rice through *Agrobacterium* and showed that efficiency of activation tagging is high in the genes within the 7Kb of the inserted double 35s activators and also characterise the dominant mutant caused by enhanced expression of CYP₇₂₄B1/D11. We created a T-DNA plasmid with four tandem copies of the CaMV35S activator, which boosts the expression of neighbouring genes on either side of the carrier plasmid. In *Arabidopsis thaliana*, [15] were successful in introducing the four tandem CaMv35S.

Our goal was to create a binary vector that boosts the expression of nearby genes while also determining the gene expression profiles of genes using the 4XcaMV activator. The pSB11-bar vector construction was based on the strategy developed by Ruvkin and Ausubel [16]. The 4XcaMV35S activator and GFP reporter genes were cloned into the pSB11 bar vector's specific restriction site by Hind 111 digestion, and the plasmid was replicated in *E.coli* DH5 cells before being converted into *Agrobacterium* via triparental mating [17].

MATERIALS AND METHODS

Construction of pSB11 Bar Vector

The spectinomycin resistance gene is present in 6323 kb of the super binary vector pSB11 utilised for plant transformation. The binary vector pSB11 exhibits high frequency of transformation rates owing to having the additional virulence genes in the vector backbone and having the increasing probability of producing transformed cells on the cotyledonary nodes [18]. The pSB11 bar vector was prepared by using our method described earlier by Srinivas et al., [19].

Transformation

Plasmid was isolated from transformed DH5 cells using an alkaline-lysis method [20]. To confirm the presence of GFP, bar gene, and 4x activator the PCR reaction was performed using primers for

Activator, GFP, NOS and BAR genes. Five different reaction mixtures, were prepared: 1 with both forward and reverse primers for activator, second with both forward and reverse primers for GFP; third with GFP forward and Nos reverse primers; fourth reaction with Bar forward reverse primers used and the last reaction with Bar forward and Nos reverse primers (Table 1). PCR was performed at 95°C for initial denaturation for 300 sec and 30–40 cycles (denaturation the plasmid for 15–30 sec, 45–68°C for annealing step for 60 sec, 68°C for elongation step 60 sec) and 72°C for final extension for 5 mins and finally at 4°C for hold up the reaction and final products were run on 1 % agarose gel electrophoresed [21].

Table 1. List of forward and reverse primers used.

Activator Forward	5' CAAAGGGTAATATCGGAAACC 3'
Activator Reverse	5' TCACATCAATCCACTTGCTT 3'
Gfp Forward	5' TGGTGTTCAATGCTTTTCAA 3'
Gfp Reverse	5' CAGGTAATGGTTGTCTGGTA 3'
Bar Forward	5' AACGACGCCCGCCGACA 3'
BAR REVERSE	5' GGTGACGGGCAGGACCGGAC 3'
NOS REVERSE	5' ACATAGATGACACCGCGCGC 3'

Preparation of Agrobacterium Strain and Confirmation of Vector Map

Two to three days before the transformation, streak out DH5α cells containing recombinant plasmid on YEP solid media containing antibiotic and grow the culture at 29°C for 2–3 days. Recombinant bar vector was transformed into Agrobacterium strain EHA105 through triparental mating by using pRK helper plasmid. Transformed EHA 105 cells are identified by using tetracycline 10mg/L, Rifampicin 10 mg/L and spectinomycin 50mg/L in YEP medium. Isolated plasmid from the selected colonies was subjected to restriction digestion with Hind III, Sac I and Xho I. Restriction digestion reaction was setup by adding 15 µl nuclease free water, 2 µl Fast Digest buffer, 2 µl plasmid DNA and 1 µl FastDigest enzyme into a vial then mix gently and spin down the content and incubate at 37°C in water thermostat for 15 mins and stop the reaction by inactivating the enzyme at 80°C for 10 mins for HindIII and xhoI and 65°C for 20 mins for SacI enzyme. The products were electrophoresed on 1% agarose gel to confirm the restriction map T-DNA construct [21].

RESULT

The vector is important in the creation of transgenic lines because it can introduce new features or disclose the properties or functions of unknown genes. The greatest tool for identifying genes is activator tagging. We created a vector by cloning a 1.4-kb segment containing a CaMv35S (4xAc) activator tetramer and a 1.8-kb GFP cassette into the pSb11 bar vector at the Hind III site. The vector was deployed into E.coli DH5 cells using a calcium phosphate mediated transformation approach for plasmid vector amplification. An alkaline lysis technique was used to acquire the plasmid, and PCR amplification was used to validate its presence in E.coli DH5 cells. The 4X activator, Bar gene, Gfp gene, Gfp gene with NOS gene, and Bar gene with NOS gene were all amplified using different primers in the PCR experiment. The pSB 11 bar plasmid vector was successfully transformed into E.coli DH5 cells, as shown in Figure 1.

The recombinant clone comprising varied expression units of the T-DNA construct was mobilised into Agrobacterium EHA 105 cells through tri parental mating, and the resulting super binary vector was named pSB111-Bar-4XAc-GFP. To select against modified agrobacterium colonies, antibiotics on YEP medium are utilised. Figure 2 shows recombinant Agrobacterium colonies on YEP media. Tetracycline (10 mg/L), Rifampicin (10 mg/L), and spectinomycin 50mg/L in YEP medium are used to identify. The alkaline lysis approach was used to isolate plasmids from transformed cells that were selected against antibiotic resistance genes. Restriction digestion was performed on the acquired plasmid using enzymes such as Sac I, Hind III, and XhoI. The Sac I enzyme [22] was isolated from *Streptomyces achromogenes* and recognises the sequences 5'....GAGCTC....3' and 3'....CTCGAG 5', creating 3' protruding ends. The SacI enzyme digests the pSB111 bar-4XAc-GFP plasmid, resulting in

a 1.8 kb GFP cassette fragment. HindIII, a type II restriction enzyme isolated from *Haemophilus influenzae*, recognises the palindromic sequence. By hydrolysis in the presence of the cofactor Mg^{2+} , 5'....AAGCTT....3' and 3'....TTCGAA 5' form 5' overhangs sticky ends [23]. The enzyme cleaves the plasmid pSB 111 bar-4XEn-1.4kb GFP's 4XEn fragment. By creating the 5' protruding ends, the XhoI enzyme obtained from *Xanthomonas holcicola* recognises the double standard sequences 5'....CTCGAG....3' and 3'....GAGCTC...5'. [24]. The XhoI restriction enzyme cleaves the bar gene in pSB111-bar-4X-Ac-GFP, which is roughly 560 basepairs long (Figure 3). The restriction map of the T-DNA construct of the pSB111-Bar-4X-Ac-GFP plasmid was validated using the aforesaid digestion procedures. The restriction map of the plasmid pSB111-Bar-4XAc-GPF is shown in Figure 4.

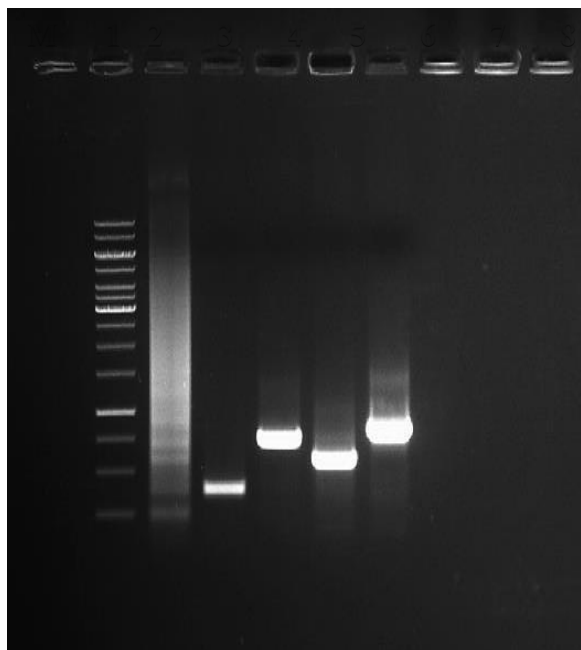


Figure 1. The figure shows PCR amplify products of vector plasmid which is isolated from *E. coli* DH5 α cells the lanes indicates that Lane M: 1 kb ladder; Lane 1: Activator forward and reverse primers; Lane 2: GFP forward and Reverse primers; Lane 3: GFP Forward and Nos Reverse primers; Lane 4: Bar Forward and Reverse primers; Lane 5: Bar Forward and Nos Reverse primers. From the bands images it was confirmed that the presence of pSB 11 bar vector.



Figure 2. This figure represents transformed colonies of *Agrobacterium* EHA105 cells. The bacterium contains T-DNA construct pSB111-Bar-4XAc-GFP vector.

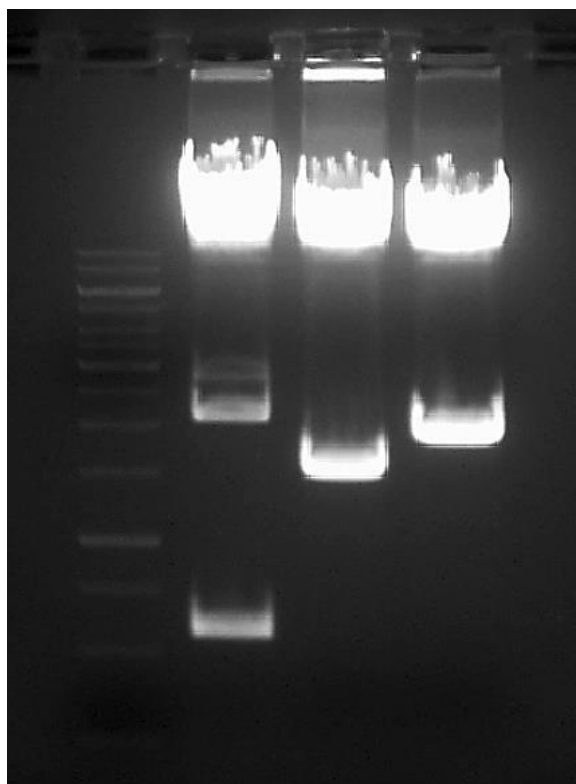


Figure 3. The figure indicates Lane M: 1kb ladder; Lane 1: digestion with Xho (560 bp) indicates Bar gene; Lane 2: digestion with Hind III (1.4 kb) indicates 4xAc; Lane 3: digestion with SacI (1.8 kb) indicates GFP cassette.



Figure 4. This figure represents T-DNA construct by harbouring different expression units and it is named as pSB111-Bar-4XAc-GFP vector and it shows the restriction map of Hind III, SacI and xhoI restriction enzymes.

CONCLUSION

Many strategies, including as mutagenesis, transposons tagging, and deletion mutagenesis, have been developed to exploit the function of genes in the genome. Activator tagging is the most suited approach for identifying and characterisation of genes in the genome of species. Up to 7 kb away from the insertion site, the activator tag vector is responsible for activating the genes. The activator tag vector is an excellent tool for identifying novel, non-expressive genes, genes expressed just during a specific time of life, and primitive genes. It produces overexpression of genes or ectopic expression of endogenous genes. It promotes gain of function of genes rather than loss of function of genes. In this study, we developed the plasmid pSB 11Bar vector, which contained the 4X-Ac gene activator and GFP selectable markers and was transformed into the *Agarobacterium* EHA105, which was resistant to tetracyclin, Rifampicin, and spectinomycin. The 4X-Ac acts as an activator for genes, causing them to activate four times as much as they would normally. The location of the 4X-Ac insertion has a significant impact on gene expression. Many genes in a species are not expressed, are expressed seldom, or are not expressed at all. This activator tagging method can help uncover the precise function of these

genes. Many crops are affected by biotic and abiotic stresses such as humidity, temperature, salt stress, and different diseases caused by bacteria, fungi, and viruses. In order to efficiently deal with specific pressures, plants develop stress resistance by modifying their DNA. It has a possibility to resist stress by expressing genes that are effective against both biotic and abiotic stress using this vector. Crop yields may be improved as a result of the designed vector.

Finally, *Agrobacterium* is commonly utilised in plant genetic engineering to create transgenic plants. The bacterium with the binary plasmid pSBIII bar vector strain is ready for infection and transformation of plants. For decades, the *Agrobacterium* transformation process has been utilised to produce transgenic crops by efficiently transferring genes in a variety of agronomically important plant species such as maize, rice, and soybean johar.

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