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Direct Cloning Approach for Expression of an Anti-cucumber Mosaic Virus Single-chain Variable Fragment in Plant

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Abstract: An anti Cucumber Mosaic Virus (anti-CMV) single chain variable fragment (scFv) was constructed into a plant expression vector using self-designed primers. Initially, sequence information of the scFv was obtained through automated sequencing. Based on the sequence information, two primers with appropriate restriction endonuclease sites were designed to facilitate the clone construction. This technique allows insertion of the desired gene to replace the *GUS* second exon in pCAMBIA 1301, without the need to form a complete cassette including promoter and termination signal prior to cloning into the vector. After insertion of the construct into the plant system through Agroinfection, the foreign protein, anti-CMV scFv antibody was expressed in tobacco plants.

Key words: scFv, primer, pCAMBIA 1301

INTRODUCTION

This research was carried out using variable fragment of anti-CMV antibody as the insert DNA for cloning. The expressed recombinant antibody from tobacco plant was selected for affinity to cucumber mosaic virus^[1]. Cucumber Mosaic Virus (CMV) is a member of the cucumoviruses. It exists as a number of strains and has a single-stranded positive sense RNA genome^[2]. This virus has a very broad natural host range^[3].

Variable fragment (Fv) is the smallest unit of immunoglobulin. Due to its smaller size, Fv fragment is easier to manipulate than a whole antibody molecule and it has become very useful for many applications^[4-6]. Single-chain variable fragment (scFv) refers to recombinant antibody fragment consisting of only the V_H and V_L domains connected by a linker^[7-9]. Overall, the construction of an anti-CMV scFv was described previously^[1].

In this study, a plant expression vector, pCAMBIA-1031^[9] as shown in Fig. 1 was chosen for rapid construction. This vector contains two CaMV promoters which control the antibiotic resistant gene and *GUS* gene expression separately. The *GUS* second exon is flanked by un-repeated *Nco* I and *Pml* I restriction endonuclease sites, therefore cloning procedures can be simplified by removal of the *GUS* gene using *Nco* I and *Pml* I restriction

enzymes, followed by insertion of the desired foreign gene in-frame into the particular region.

Experimental procedures: Modification of the pCAMBIA-1301 plant expression vector was carried out by removing the *GUS* A second exon-His6 using *Nco* I and *Pml* I restriction endonucleases. Based on the sequence of the anti-CMV scFv^[1], 2 primers, SCFVF and SCFVR (Table 1) were designed. The primers were used to amplify the scFv DNA fragment through PCR prior to cloning into *Nco* I/ *Pml* I digested pCAMBIA-1301. Double digestion of 2 µg of amplified DNA fragment was carried out in 20 µL reaction volumes containing 10 U of *Nco* I and *Pml* I (New England Biolabs), 1X buffer 4 and the appropriate amount of sterile distilled water. The reaction was performed at 37°C for 2 h. Two µg of the anti-CMV scFv PCR product were also digested using the same restriction enzymes. Ligation of scFv PCR product

Table 1: Cloning Primers Used for Amplification of Anti-CMV ScFv

Primers and sequence

Forward primer: SCFVF

5'-**CATG** CCATGGATGCATCACCATCACCATCACATGGTGCA
GCTGCAGGAG-3'

Reverse primer: SCFVR

5'-**CATG** CACGTGTCAGTCGACCTGTGCATCGTCGTCCTT-3'

Nonsense sequence is indicated in black block, *Nco* I restriction endonuclease site is italicised, *Pml* I restriction endonuclease site is in bold text, His6x-tag sequence is underlined, stop codon is in smaller font phase

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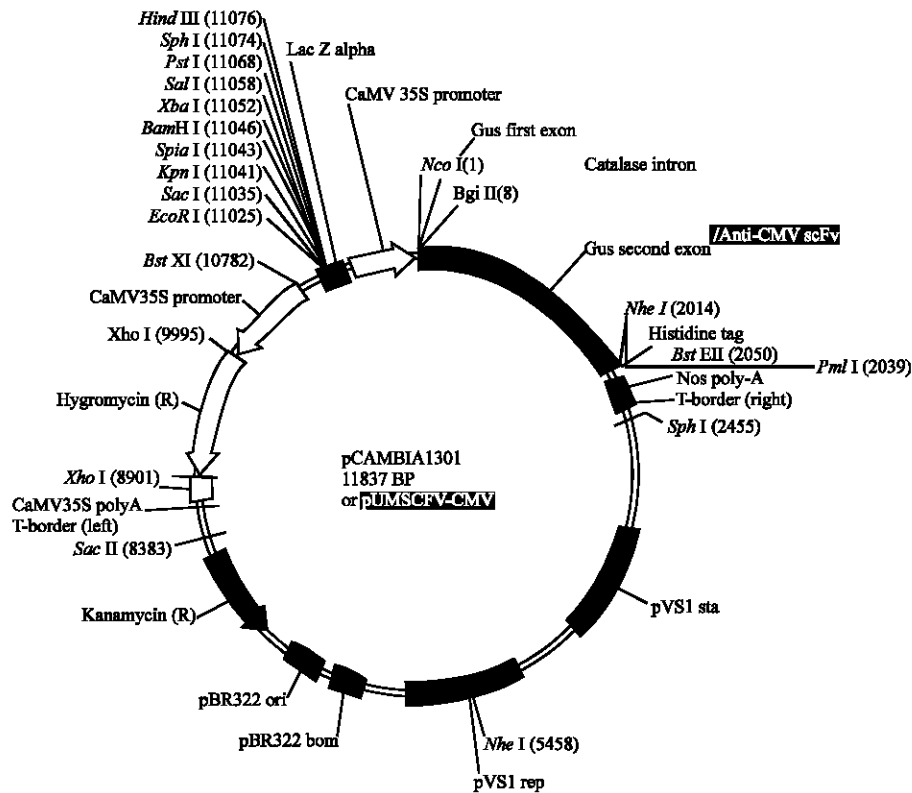


Fig. 1: Cloning of an anti-CMV scFv DNA fragment into plant expression vector, pCambia 1301. This diagram shows the outline and restriction endonuclease sites of the vector. After double digestion using *Nco* I and *Pml* I endonucleases, the *GUS* second exon cassette was removed and replaced with an anti-CMV scFv DNA fragment to form pUMSCFV-CMV1 construct as shown in black block.

pCambia DNA fragment was carried out using 3U of T4 ligase (Promega) in a total volume of 20 μ L containing 1X ligation buffer and an appropriate amount of sterile distilled water. The ligation was performed overnight at 16°C to form the pUMSCFV-CMV1 construct (Fig. 1). Overall, the construction strategy used in this research is shown in Fig. 2. After that, the construct was introduced into tobacco plants through Agroinfection and followed by protein expression study.

RESULTS

When the anti-CMV scFv construct was amplified by PCR using SCFVF and SCFVR primers, \approx 797 bp DNA fragments were obtained as shown by black arrow in Fig. 3. The fragment was then digested with *Nco* I and *Pml* I resulting in smaller size DNA fragments as indicated by the white arrows in the same figure. Concurrently, pCambia-1301 plasmid was successfully digested with the same restriction endonucleases. The digestion resulted in 2 fragments where the 2046 bp fragment is the *GUS* second exon-His6 and the 9038 bp fragment is the

digested pCambia-1301 vector that would be used in the cloning experiments (Fig. 4). Ligation of both the digested PCR product and the pCambia-1301 vector was successfully done and the construct was transformed into competent *E. coli* DH5 α . Analysis of the construct was carried out by digesting DNA using *Nco* I and *Pml* I restriction endonucleases resulting in two expected fragments where the \approx 9038 bp fragment is the pCambia-1301 vector and the \approx 785 bp is the inserted anti-CMV scFv DNA fragment as shown in Fig. 5. Further expression of the scFv antibody in plant was carried out by western blot and the result is shown in Fig. 6.

DISCUSSION

In this study, we focused on an alternative construction technique for cloning of novel foreign protein gene into plant expression vector, followed by study of protein expression in plants. An anti-CMV scFv antibody constructed in a previous study was used in this study^[1]. The antibody produced has potential both to modify the intrinsic properties of the plant thus conferring

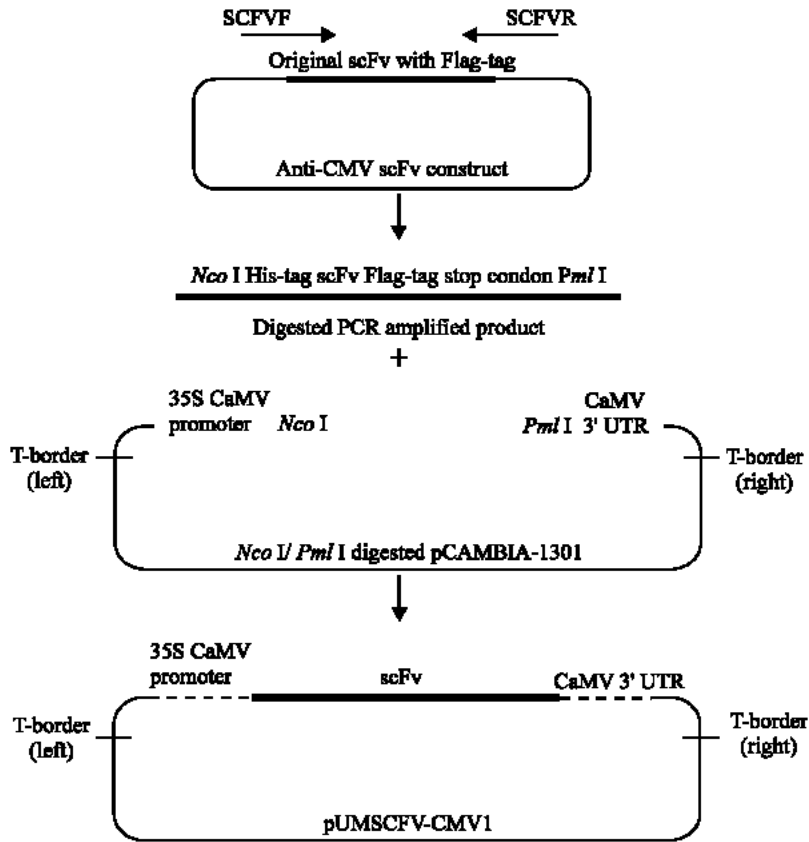


Fig. 2: The flow chart of Anti-Cucurbit Mosaic Virus Single-Chain Fv Antibody Gene into Plant Expression Vector. Amplification of the anti-CMV scFv DNA fragment was carried out using the SCFVF and SCFVR primers. The PCR product and pCAMBIA-1301 vector were subjected to *Nco*I and *Pml*I digestion followed by ligation to form pUMSCFV-CMV1 construct

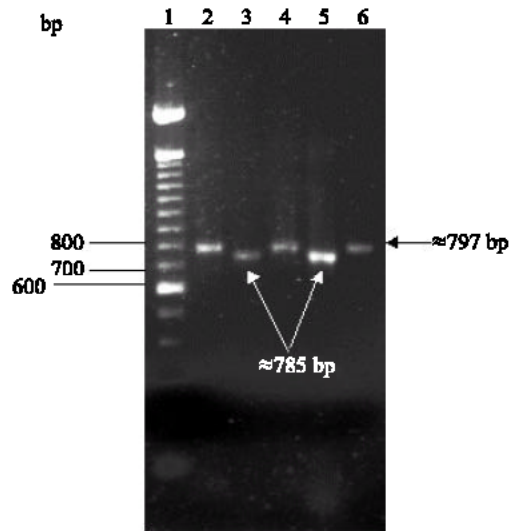


Fig. 3: Undigested and Digested ScFv PCR Product. One hundred bp ladder (Gibco-BRL) (lane 1), scFv PCR samples (lane 2, 4 and 6) and *Nco*I/*Pml*I digested scFv PCR samples (lane 3 and 5) were separated on a 2% agarose gel and stained with ethidium bromide. The black arrow shows that the ≈ 797 bp PCR products in the appropriate lanes while the white arrows show the ≈ 785 bp *Nco*I/*Pml*I digested scFv PCR products

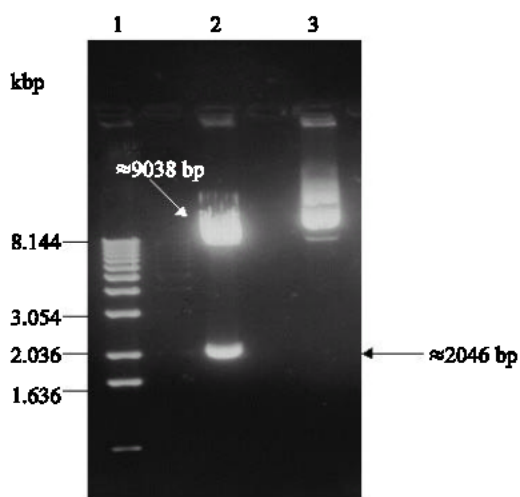


Fig. 4: Undigested and Digested pCAMBIA-1301 vector. One kbp DNA ladder (Gibco-BRL) (lane 1), *Nco* I / *Pml* I digested pCAMBIA-1301 plasmid sample (lane 2) and undigested pCAMBIA-1301 plasmid sample (lane 3) were separated on 1% agarose gel followed by ethidium bromide staining. An approximately 2046 bp *GUSA* exon-His6 fragment was digested out from the plasmid as shown by the black arrow while the ≈ 9038 bp fragment as shown by a white arrow is the desired cloning vector with specific cloning sites

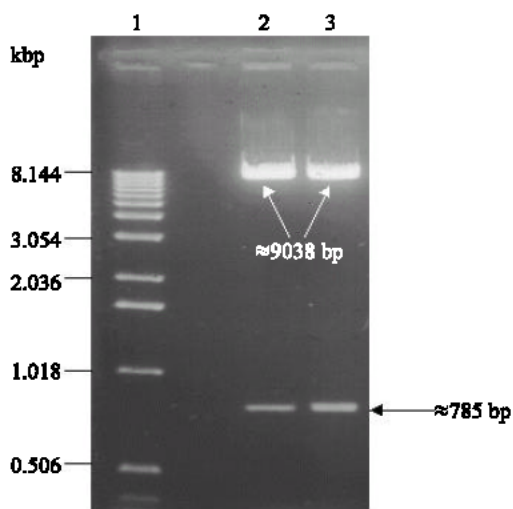


Fig. 5: Digested pUMSCFV-CMV1 construct. One kbp DNA ladder (Gibco-BRL) is shown in lane 1. pUMSCFV-CMV1 was digested using the *Nco* I and *Pml* I endonuclease resulted in 2 fragments where the ≈ 9038 bp fragment as shown by the white arrow is the digested pCAMBIA-1301 while the ≈ 785 bp fragment as shown by the black arrow is the anti-CMV scFv fragment (lane 2 and 3)

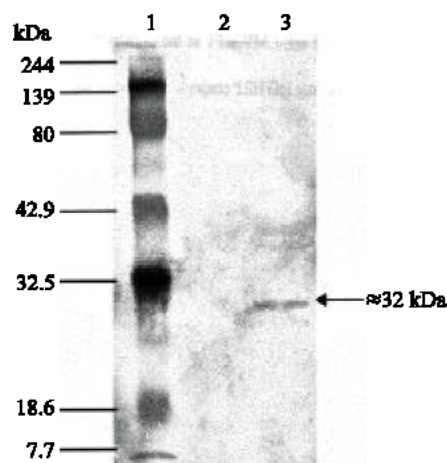


Fig. 6: The kaleidoscope protein molecular weight standard (lane 1), ≈ 2 μ g of total protein isolated from wild type tobacco leaves as a negative control (lane 2) and total protein isolated from transgenic tobacco leaves (lane 3) were separated on a 12% SDS-PAGE gel and blotted to a nitrocellulose membrane. Detection was carried out using anti-FLAG as the primary antibody, followed by goat anti-mouse IgG HRP conjugated antibody. The ≈ 32 kDa scFv antibody was detected in the transgenic leaf but absent in the negative control

a novel form of resistance or tolerance to the targeted disease and can also be used to produce a diagnostic reagent.

Initially, the desired anti-CMV scFv DNA fragment was successfully cloned into a plant expression vector, modified pCAMBIA-1301 to form the pUMSCFV-CMV1 construct. This study outlines a systemic and straightforward approach for construction using self-designed primers to incorporate appropriate restriction sites for cloning and a convenient hexahistidine tag (His6-tag) selection marker. The His6-tag was incorporated into the N-terminal of the scFv in this study based on the similar strategy to that by Ramirez *et al.*^[10] to avoid affecting the binding affinity of the scFv. Goel *et al.*^[11] showed that construction of the His-tag on the C-terminal position of CC49 scFv adversely affected the binding properties of the construct. His-tag is useful to facilitate desired scFv purification by affinity chromatography in the future.

pCAMBIA-1301 was used in this study because it contains origins of replication functional in both *E. coli* and *Agrobacterium*. Kanamycin resistance gene and hygromycin resistance gene for selection of transformed bacteria and transgenic plants are the convenient features of this vector. *Agrobacterium*-mediated transformation

and a plant binary expression vector system were used in this study as it had been well proven to generate stable transformants in tobacco plants^[12].

After transformation, initial selection of the cells with the integrated gene was carried out on antibiotic selective media. The expression of the desired 32 kDa scFv antibody in transgenic plants was confirmed by western blot analysis (Fig. 6).

Overall, the scFv antibody production was successfully demonstrated in plants. This rapid construction of foreign protein gene into a selected plant expression vector allows the bypass of cassette formation and has given promising expression results in this study. Besides that, it shortened the time to produce transgenic plants expressing foreign protein.

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