Subcloning of VHH Single Domain Antibody & its transformation into Tobacco mediated Using *Agrobacterium* (C58GV3101)

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Abstract: - Functional VHH single domain antibody lacking light chains occur naturally in Camelidae.The single domain nature of VHH gives rise to several unique features as compared to antigen–binding derivatives of conventional antibodies. The level of expression in *E.coli* was found to be too low for therapeutic purposes so we have to use the other production systems like plants. Several Plants are the facile and economic bioreactor for large-scale production of industrial and pharmaceutical agents like proteins and antibodies, and tobacco was our target plant because of large scale production and many other advantages. In this study we have subcloned VHH gene into pBI 121 using phasmid pCANTAB5E. The new construct was used to transform the *Agrobacterium* strain C58GV3101. Transgenic tobaccos (NC25) Plants were then developed by introducing VHH gene under the control of CaMV 35S promoter. The presence of the VHH antibody gene in the plant genome was verified both by selection of the resistant plants on selective media and by PCR analysis.

Key-words: VHH antibody fragment; subcloning; Plantibodies; Transgenic tobacco; Bioreactor; MUC1

1 Introduction

Antibodies have been used as diagnostic or therapeutic agents in vivo as well as ex vivo for the last two decades. Their clinical applications are evident in treatment of diseases such as cancer, transplantation, autoimmunity and cardiovascular disorders. They have been specifically used in cancer therapy because of their high specificity for tumour antigens and low cross-reactivity with normal cells. In some isotypes such as camelids from the old world (camels, dromedaries) or the ones from the new world (llamas, vicuna) the L chain is missing [8] Furthermore, their H chain is devoid of the CH1 domain due to an unconventional splicing event during mRNA maturation. Therefore the antigen-binding fragment of the heavy-chain antibody consists of a single domain referred to as, VHH that replaces a four-domain Fab fragment in the immunoglobulin structure [13]. Besides the advantages of easy cloning (single gene) and selection from in vivo matured library it have other technological, physiochemical and functional advantages, such as close homology to human VH fragments, high expression yield, highly soluble and the generation of antigen-specific, high affinity binders [14].VHH domain has been expressed in several different host–vector systems including yeast [18] and E.coli [19]. Recently

camelied single domain antibodies have been used to target MUC1 antigens in breast cancer [25]. However, these expression systems have several limitations that hinder maximum output of biologically active and safe therapeutic agents. Some of these limitations that have been reported are: (1) formation of inclusion bodies in bacteria, (2) formation of non-native proteins having different biological activities in yeast, (3) low transgene expression levels, (4) transgene induced instability of certain cell lines in mammalian cell cultures, and (5) contamination of animal-based products with human pathogens. Such shortcomings invite alternative methods of production to ensure the safety and economical benefits of recombinant therapeutic proteins [3]. Plant-based systems are increasingly used for the production of recombinant proteins including antibodies. Plant-based systems have several advantages over the other production systems, such as the ability to carry out necessary post-translational modifications not available in bacterial systems, as well as greater safety and lower production costs compare to animal- based systems. Plant-based technology has been recently reviewed, with full description of commonly used plants [23]. Processing of transgenic crops would require relatively little capital investment, making the commercial production of biopharmaceuticals an exciting prospect. It has been estimated that the cost of producing recombinant proteins in plants could be 10 to 50 fold lower than producing the same protein in E. coli or mammalian cells. Several proteins, enzymes and antibodies have been produced in plants and used in clinical trials, with a prospect of commercial exploitation [12].

2 Materials and methods

2.1 Sub-cloning

The phasmid vector pCANTAB5E containing the VHH coding sequence that was partially optimized for expression in competent E. Coli TG1 was obtained from Tarbiat Modares University, Department of Medical Biotechnology. The VHH encoding region was amplified from pCANTAB5E phasmid DNA using the primers Back: 5′-GGAAATTCGAGCTCTTAGTGAGATGGTG AC-3′ and Forward: 5′TCTAGAGGATCCTA AACAATGGTCCTGCTACAGTCA-3′ Each PCR mixture was prepared in a final volume of 50 µl containing 50 ng pCANTAB5E phasmid DNA template, 50 pM forward primer, 50 pM of the corresponding backward primer, 40pM dNTPs, 25 mM MgCl2, 1.25 U Taq DNA polymerase (Cinnagen) and 5 µl 10×PCR buffer II (Cinnagen). Hot start PCR was performed at 95 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 45 s and elongation at 72 °C for 1 min. The reaction was completed by a final extension time at 72 °C for 1 min. The amplified variable regions were purified by electrophoresis on a 1.5% agarose gel and subsequently extracted with an AccuePrep Gel Extraction Kit (Bioneer) according to the manufacturer's instructions. Amplified DNA was cloned into T/A vector (pTZ57R)using the InsT/A clone PCR product Cloning kit from Fermentas. pTZ57R plasmid was cut with *BamH* I and *Sac* I (Roche Applied Science). After another electrophoresis on a 1.5% agarose gel, the variable regions were excised and extracted with the AccuPrep Gel Extraction Kit (Bioneer). The fragment of interest was ligated with the correspondingly cut vector pBI 121 in a 10 µl volume containing 50 ng vector DNA, threefold molar excess of the PCR product and 5 U of T4 DNA ligase (Fermentas) for 16 h at 14 °C. E. coli TG1 cells (50 µl) were transformed with 2 µl of the ligation product via Cacl2 method. One milliliter of prewarmed SOC medium [21] was immediately added, and the cells were grown at 37 °C for 1 h shaking with 250 rpm. Cells were plated on LB agar medium containing Kanamycin (25 µg/ml) and incubated overnight at 30 °C. Colonies were picked for plasmid extraction using the Accuprep Plasmid Extraction Kit (Bioneer). The engineered pBI 121 plasmids were used to transform the *Agrobacterium* strain C58GV3101 using a Freeze and Thowing standard protocol [21].

2.2 Transformation

Axenic plants of tobacco (*Nicotiana tabaccum* var.*NC25)* maintained under in vitro growth conditions at 26 °C and 16 h photoperiod in the lab. For tobacco transformation, *Agrobacterium* cells were used for transformation. Leaf explants of in vitro-grown were inoculated with Agrobacterium in a YEP liquid medium for 10 min. For effective transformation, the explants were placed in a co-culture medium containing 2.0 mg/l of 2, 4-D. Two days later, the explants were transferred to the regeneration medium (MS medium, supplemented with 2.0 mg/l BAP, 0.01 mg/l NAA, 200 mg/l cefatoxim, and 100 mg/l kanamycin). The explants were transferred to the fresh medium at 2 weeks intervals. As a control, non-inoculated explants were cultured in the same medium without hormones and antibiotics. The induced shoots were then dissected from the explants and transferred to MS medium containing cefatoxim (200 mg/l) for Agrobacterium elimination and kanamycin (100mg). This procedure also modified to use different old tobacco tissues by Dadmehr (unpublished data).

2.3 PCR analysis

After rooting of the regenerated tobacco plants on free antibiotic medium, they were transferred into the greenhouse and maintained to maturity. Genomic DNA from 200 mg each of non-transgenic plants as negative control and all putative kanamycin resistant plants was extracted from transgenic plants according to Albani et al. (1992). Transformed and control plant genomic DNA was used as a template to detect the VHH gene by Polymerase chain reaction (PCR) under the conditions that were described before and with specific primers. The 400 bp amplified DNA fragments were electrophoresed on a 1.5% agarose gel and visualized by staining with EtBr.

3 Results

3.1 Construction and transformation of vectors

Single domain antibody gene of the pCANTAB5E was subcloned into the expression vector pBI121 .The transfer of this gene is schematically shown in Fig 1. The isolation and PCR amplification of the gene encoding the VHH antibody was performed using pCANTAB5E phasmid DNA as template. Targeting the restriction sites BamH1 and Sac1 in pTZ57R plasmid facilitated subcloning. New construct (plasmid) was transformed into TG1 E-coli and the colonies were appeared on the kanamycin containing plate. After the extraction, pTZ57R and pBI121 plasmids were cut by BamH1 and Sac1 restriction enzymes. Electrophoresis showed 400 bp bands from pTZ57R and 12000 bp and 1900 bp GUS removed gene from pBI121.The amplified 400bp and 12000bp bands were gel-purified from agarose . During the ligation, VHH gene inserted into pBI121 plasmid and this plasmid was transferred to Agrobacterim strain C58GV3101. When the leaf explants were inoculated with Agrobacterium immediately after excision, shoots on the cut edges of the explants were observed in the presence of 100mg/l kanamycin and 200mg/l cefatoxim after two weeks. The putatively transformed shoots were excised when they were about 1 cm tall and transferred to a shoot elongation medium containing cefatoxim and kanamycin.Consequently shots were transferred to rooting media and after hardening to the greenhouse. The genes that had been stably integrated into the plant genome were translated under the control of CaMV35S, resulting in the expression of the VHH gene and the growth of transformants on medium supplemented with kanamycin.

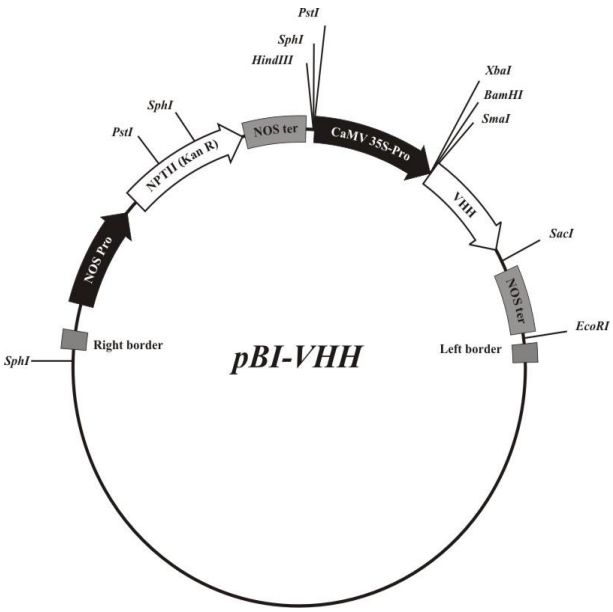

Fig.1, Schematic representation of pBI-VHH construction .VHH gene inserted between CaMV 35S promoter and NOS terminator.

3.2 Expression of VHH antibody fragment in plants

We obtained more than 50 kanamycin-resistant putative transformants and carried out further analysis of VHH gene expressions on selected transgenic plants. Untransformed tobacco plants that had been regenerated from leaf discs without kanamycin selection, were used as negative controls. PCR analysis was carried out as the first method to confirm the transgenic nature of the regenerated plants. The presence of VHH DNA in the genomic DNA isolated from regenerated tobacco was confirmed. Transformants were detected by PCR amplification of inserted VHH with specific primers. The expected 400 bp VHH bands were found in the transformants (Fig. 2). No DNA product was detected in untransformed control plant DNA. The same fragment was amplified using plasmid pBI 121 as our positive control.

4 Discussions

Expression of the first recombinant antibody in tobacco plant, opening the field of the expression of recombinant antibodies as a real alternative to eliminate many constraints of monoclonal antibody production in bioreactors (Hiatt et al 1980s). Expression of antibodies in plants is being studied for their potential uses in biotechnology.Camelidae are known to produce

Fig.2, Comparison of Transformed and Non-transformed leaves on selective media containing 100 mg /l kanamycin and 250 mg/l cefatoxim.

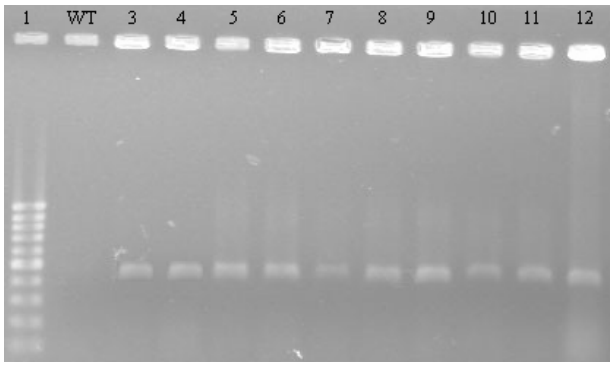

Fig.3, Agarose gel electrophoresis of VHH amplification products from transgenic plants. Lane 1:100bp ladder; lane 2: wild type tobacco; lanes 3-12 transgenic tobacco plants

Immunoglobulin (Igs) devoid of light chains and constant heavy-chain domains (CH1). Antigen-specific fragments of these heavy-chain IgGs (VHH) are of great interest in biotechnology applications (Muyldermans, 2001) .VHH single domain antibody fragments against MUC1 antigen have been produced, but exclusively in bacteria and yeast. We have described here for the first time, the expression of a MUC1 specific VHH antibody in plants. To facilitate immunohistochemical, biochemical, and bioassay investigation large amount of VHH is required therefore we use the CaMV 35S promoter which is very strong constitutive promoter, causing high levels of gene expression in dicot plants. Before that we use the T/A cloning vector (PTZ57R) because we want to take advantages of inserted MCS (Multi Cloning Site) which is beneficial for cloning and also M13 primers around the MCS that

facilitate the gene sequencing using this plasmid resulting PCR product ligated into a linear vector with a 3´ terminal 'T' or 'U' at both ends. Tobacco as a laboratory bench model was our target plant because of its large scale production and as compared to other plants represents a potentially safer production vehicle for human proteins from the standpoint of containment. In an effort to study the safety and reproducibility of VHH single domain antibody production in plant-based systems, a transgenic tobacco plant expressing this antibody was grown as a leaf disk culture on selective medium. Two weeks later, the plant cell cultures were initiated in selective medium and examined for VHH antibody production. Expression of the VHH appeared to remain constant throughout the growth periods, with no effect on the growth rate. This VHH appears to be an intrinsically stable molecule, able to accumulate in the plant cell cytosol and to maintain its functionality. Transient and stable plant system used here to express VHH gene, has the same advantages and limitations that have been already described in literature for similar cases. Various methods have been recently applied to produce and select functional antibodies. More studies are required to use the different subcloning procedures by different plant vectors, optimize the expression and production of VHH gene in plants and the extraction/purification procedures that have substantial impacts on the final outcome.

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