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Full Length Research Paper

Expression of biological active VHH camelid single domain antibody in transgenic tobacco

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Functional VHH single domain antibody lacking light chains occur naturally in Camelidae. The single domain nature of VHH gives rise to several unique features when compared to antigen-binding derivatives of conventional antibodies. The level of expression in *Escherichia coli* was found to be too low for therapeutic purposes. Therefore, there is a need to examine other production systems such as plants. Several plants are the facile and economic bioreactor for large-scale production of industrial and pharmaceutical agents like proteins and antibodies. Here, we have selected tobacco as the host plant because of large scale production capability and many other advantages such as greater safety and lower production costs when compared to animal-based systems. In this study, we have subcloned VHH gene into pBI121 using phasmid pCANTAB5E. The new construct was used to transform the *Agrobacterium* strains C58GV3101 and LBA4404. *Agrobacterium* strain C58GV3101 showed a higher virulence on leaf disks of *Nicotiana tabacum* (NC25). Transgenic tobacco 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 by PCR analysis and Southern hybridization experiments. Northern blot analysis showed that the genes coding for the VHH could be expressed in tobacco plants. Three lines of transgenic plant that expresses high levels of mRNA were screened in a further analysis. The expression of VHH was then observed in transgenic plants by ELISA using the specific antibody developed, the results showed three to five folds higher than non-transgenic tobaccos.

Key words: VHH antibody fragment, subcloning, antibodies, transgenic tobacco, bioreactor.

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 the 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. VHHs are heavy chain variable domains derived from immunoglobulins (IgG) naturally devoid of light chains such as those derived from Camelidae in previous studies (Hamers-Casterman et al., I993) 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 (Muyldermans et al., 1994). VHH molecules are about 10 times smaller than IgG molecules. They are single polypeptides and very stable, resisting extreme pH and temperature conditions. Moreover, they are resistant to the action of proteases which is not the case for conventional antibodies. Furthermore, *in vitro* expression of VHH's produces high yield, properly folded functional VHHs. In addition, antibodies generated in Camelids will recognize epitopes

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than those recognised by antibodies generated *in vitro* through the use of antibody libraries or through immunization of mammals other than Camelids (Ghahroudi et al., 1997). As such, anti-MUC1 VHHs may interact more efficiently with its target than conventional antibodies, thereby blocking its interaction with the target ligands more efficiently. Since VHH's are known to bind into unusual epitopes such as cavities or grooves (Spinelli et al., 2000), the affinity of such VHH's may be more suitable for therapeutic treatment. Besides the advantages of easy cloning (single gene) and selection from *in vivo* matured library, it has 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 (Nguyen et al., 2001). Recently, Camelid single domain antibodies have been used to target MUC1 antigens in breast cancer (Taylor-Papadimitriou et al., 2002). These fragments have been expressed in both *Escherichia coli* and *P.* pastoris (Rahbarizadeh et al., 2006). 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 (Koprowski et al., 2005). Such shortcomings invite alternative methods of production to ensure the safety and economical benefits of recombinant therapeutic proteins (Carmer et al., 1999). Plant-based systems are increasingly used for the production of recombinant proteins including antibodies (During et al., 1990; Peeters et al., 2001; Torres et al., 1999; Schillberg et al., 2005). 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 when compared to animal-based systems. Plant-based technology has been recently reviewed, with full description of commonly used plants (Hellwig et al., 2004). 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 that for producing the same protein in *E. coli* or mammalian cells (Stoger et al., 2000, 2002). Several proteins, enzymes and antibodies have been produced in plants and used in clinical trials, with a prospect of commercial exploitation (Ma et al., 2003). In this report, which is the first of its kind, we described the cloning and expression of VHH single domain antibody in tobacco plants as a first step in the development of a plant based vaccine that could produce VHH and a low-cost biomass scale-up was performed with this bioreactor.

MATERIALS AND METHODS

Construction of the expression vector pBI-VHH

The phasmid vector pCANTAB5E containing the VHH coding sequence that was partially optimized for expression in competent *E. coli* TG1 was obtained from the Department of Medical Biotechnology, Tarbiat Modares University (AY544575). The VHH encoding region was amplified from pCANTAB5E phasmid DNA using the primers back: 5´-GGAAATTCGAGCTCTTAGTGAGATGGTGAC-3´ and forward: 5´TCTAGAGGA TCCTAAACAATGGTCCTGCTACAGTCA-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, 40 pM dNTPs, 25 mM $MgCl_2$, 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 10 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 corresponding 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 $CaCl_2$ method. One milliliter of prewarmed SOC medium (Sambrook et al., 2001) 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). *Agrobacterium tumefaciens* strains C58GV3101 and LBA4404 have been previously described (Hoekema et al., 1983; Koncz et al., 1986). The engineered pBI 121 plasmids were used to transform the both earlier mentioned *Agrobacteriums* using a freeze and thawing standard protocol (Sambrook et al., 2001).

Transformation

Axenic plants of tobacco (*Nicotiana tabaccum* var.*NC25)* were maintained under *in vitro* growth conditions at 26°C and 16 h photoperiod in the laboratory. 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 BAP and 0.01 mg/l NAA. 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 free hormone MS rooting medium containing cefatoxim (200 mg/l) for *Agrobacterium* elimination and kanamycin (100 mg) as a selective antibiotic. Also, specific modified MS media were developed and used for co-culture, regeneration and rooting steps by Dadmehr (Unpublished data). When the roots were induced on the regenerated shoots, plantlets were transplanted to moist vermiculite for acclimatization. When the shoots had began to grow, the plantlets were transplanted to moist soil and the plants were grown to maturity.

PCR analysis and Southern blot hybridization

Genomic DNA from 200 mg each of non-transgenic plants as negative control and all putative kanamycin resistant plants was extracted from transgenic plants using the CTAB method (Rogers et al., 1994). 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 327 bp amplified DNA fragments were analyzed by electrophoresis on a 1.5% agarose gel stained with EtBr and observed under UV illumination. The genomic DNA also was digested with SacI to confirm the copy number of inserted T-DNAs for the VHH derived transformants. The digested DNA was electrophoresed on a 1.0% agarose gel and then transferred to positively charged nylon membrane (Roche Co., Germany) using a Turboblotter system (Schleicher and Schuell). Dig-labeled probes were generated by PCR Dig Labeling Mix (Roche Co., Germany) with the specific primer set for the VHH gene. DNA gel blots were hybridized at 50°C in a DIG easy hybridization buffer. After hybridization overnight, the membrane was washed in an SSC buffer series and then detected using the Dig Detection Kit following the manufacturer's instructions (Roche Co., Germany).

Northern blot analysis

Total RNA from the leaves of transformed plants was isolated using the RNAgents® Total RNA isolation system (Promega Co., USA) according to the manufacturer's instructions. Samples of 30 µg of total RNA were denatured with formaldehyde and formamide and fractionated in a 3.0% agarose gel using a 3-(N-morpholino) propane sulfonic acid (MOPS) buffer. The RNA was blotted to a positively charged nylon membrane, and fixed by ultraviolet irradiation. The membranes were hybridized with Dig-labeled probes using a template containing most of the coding region for VHH. After hybridization for 10 h at 68°C, the blots were washed in an SSC buffer series and then detected by means of the Dig Detection Kit according to manufacturer's instructions (Roche Co., Germany).

Quantification of VHH levels in transgenic tobaccos

Antibodies from leaves were obtained by homogenization in a blender with liquid nitrogen, and the resulting powder was resuspended in cold extraction buffer containing PBS, pH 7.2, 10 mM EDTA, 0.03% Triton X-100, 5 mM mercaptoethanol and 1 mM PMSF (1 g of fresh weight/ml). The extract was filtered and centrifuged for 20 min at 12,000 ×g and the resulting supernatant was used for the VHH protein expression analysis. ELISA plates were coated with 100 µl of coating buffer containing the sample proteins from the transgenic plants overnight at 4°C. After standing overnight, the plates were washed and blocked 2 h at RT with 5% skim milk in PBS-Tween 20. The plates were then washed three times with 0.05% Tween 20 in PBS and 100 µl of MUC1 synthetic peptide TSA-P1-24 as the primary antibody diluted at 1:1000 in PBS-Tween 20% was added to each well and allowed to react for 2 h at RT. The plates were again washed three times, and treated with 100 µl of anti-(camel) rabbit antibodies conjugated with horseradish peroxidase as the second-antibody, diluted at 1:2000 in PBS-Tween 20 for 2 h at RT. Finally, the plates were again washed four times, and 200 µl of a freshly prepared solution of ophenylenediamine dihydrochloride and H2O2 was added. Reactions were stopped by adding 2.5 M H_2SO_4. The absorbance was measured in a Multiskan ELISA Reader (Labsystems, Helsinki, Finland) using a 492-nm filter.

RESULTS

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 Figure 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 plasmid extraction, both pTZ57R and pBI121 plasmids were cut by *BamH1* and *Sac1* restriction enzymes. Electrophoresis showed 327 bp bands from pTZ57R and 12000 and 1900 bp GUS removed from pBI121. The amplified 327 and 12000 bp bands were gel-purified from agarose. During the ligation, VHH gene was inserted into pBI121 plasmid and was called PBI-VHH (Figure 1). PBI-VHH plasmid was transferred to *Agrobacterium* strains C58GV3101 and LBA4404.

Effects of two strains of *Agrobacterium* on the efficiency of transformation in tobacco

To improve the efficiency of transformation, this study examined the effects of two *Agrobacterium* strains on shoot formation from leaf disks. 168 and 162 leaf disks were infected by *Agrobacterium* strains C58GV3101 and LBA4404, respectively with a copy of pBI-VHH. All of these were transferred to plates with media containing cefatoxim. The frequency of regenerated shoots from leaf disks that were infected with C58GV3101 was higher.

Fourteen (14) transformants were obtained from leaf disks infected with the *Agrobacterium* strain LBA4404 after 3 months in culture. In this experiment, the transformation efficiency was at least 8.3%, a ratio of about 7.8-fold of C58GV3101 (Table 1). These results indicated that kind of *Agrobacterium* strains affects the efficiency of transformation in *N. tabacum*. 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 100 mg/l kanamycin and 200 mg/l cefatoxim after two weeks. The putatively

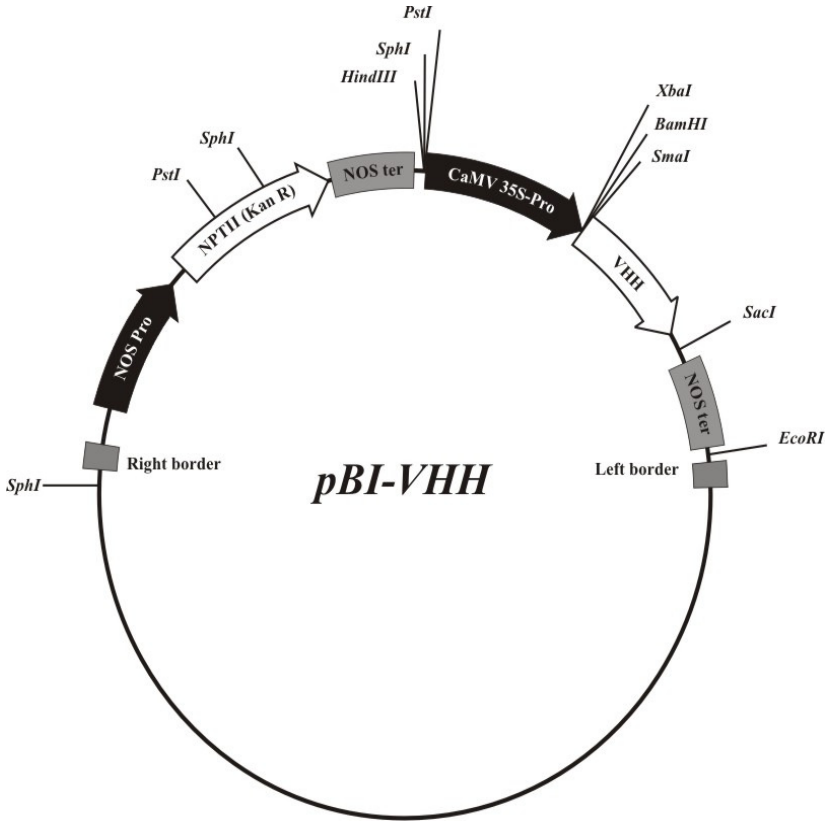

Figure 1. Schematic representation of pBI-VHH construction. VHH gene inserted between CaMV 35S promoter and NOS terminator.

Table 1. Effect of two *Agrobacterium* strains on transformation frequency.

Number of experiment	Strain	Number of leaf disk	Number of regeneration shoot	Number of transgenic shoot
EX1	C58GV3101	68	26	7
	LBA4404	52	10	3
EX2	C58GV3101	24	3	1
	LBA4404	30	0	0
EX3	C58GV3101	76	28	6
	LBA4404	80	14	0
Total	C58GV3101	168	57	14 (8.3%)
	LBA4404	162	24	3 (1.85%)

transformed shoots were excised when they were about 1 cm tall and transferred to a shoot elongation medium containing cefatoxim and kanamycin.

Consequently, shoots were transferred to rooting media and after hardenization to the greenhouse. The genes that had been stably integrated into the plant genome were transcripted under the control of CaMV35S, resulting in the expression of the VHH gene.

Expression of VHH antibody fragment in plants and molecular analysis

More than 160 kanamycin-resistant putative transformants were obtained (Table 1) 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

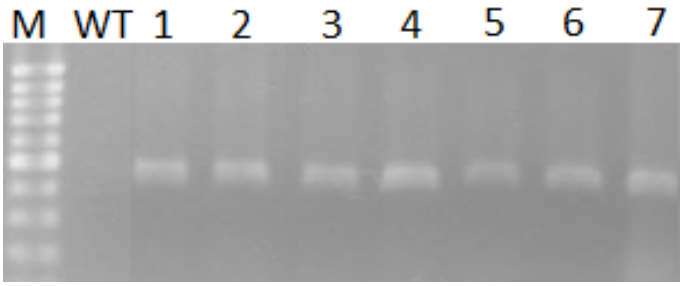

Figure 2. Agarose gel electrophoresis of VHH amplification products from transgenic plants. Lane M: 100 bp ladder; lane WT: wild type tobacco; lanes 1 to 7: transgenic tobacco plants lines 2, 3, 6, 7, 12, 18 and 19.

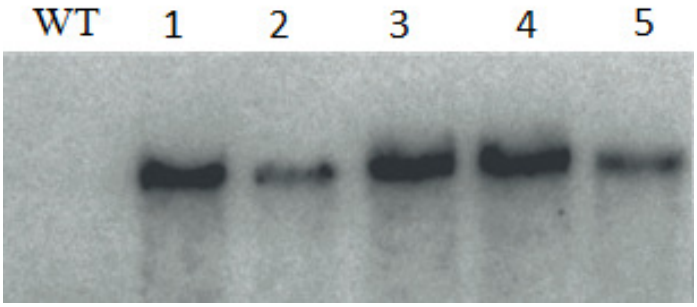

Figure 3. Southern blot analysis of transgenic tobacco lines 1, 9 and 22 for the presence of VHH gene. Lane WT: genomic DNA from non-transgenic tobacco; Lanes 1 to 5: genomic DNA from transgenic tobacco lines 2, 6, 7, 9, 12 and 18.

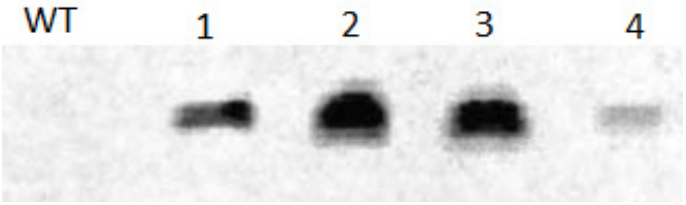

Figure 4. Expression of VHH mRNA extracted from leaf tissue. Lane WT: non-transgenic tobacco; Lanes 1 to 4: transgenic tobacco lines 2, 6, 7 and 12.

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 327 bp VHH bands were found in the transformants (Figure 2). No DNA product was detected in untransformed control plant DNA. The same fragment was amplified using plasmid pBI 121 as our positive control. Southern blot analysis of PCR positive transgenic lines (T1) was done to verify site-specific integration and to establish copy number. In order to determine the T-DNA copy number in each transformant, the DNA was digested with *SacI*, which cuts the T-DNA between VHH and the nos terminator. The probe then hybridizes to one T-DNA end fragment from each single-copy insertion, thus permitting the copy number of the VHH gene to be established. The transgene copy number as estimated by Southern hybridization ranged from one to two copies (Figure 3).

Detection of the VHH transcripts

The transformants were analyzed by hybridizing RNA samples with a labeled probe encompassing the coding region of the VHH gene. Figure 4 shows the results of an experiment, where selected transformants harboring the

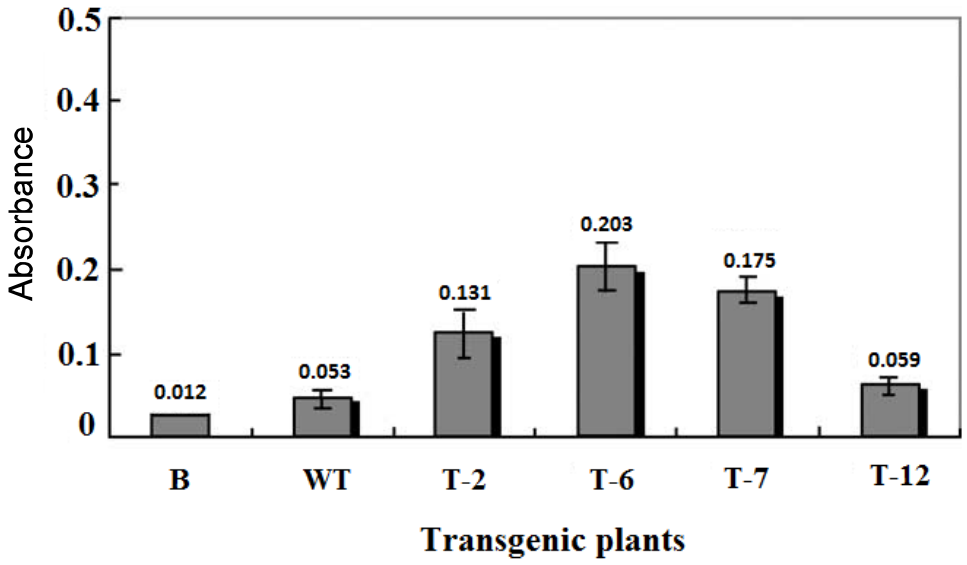

Figure 5. VHH expression levels in transgenic tobacco lines. Production of VHH in tobaccos of four independent transgenic plants (T2, T6, T7 and T12) and one wild type plant (WT) was analyzed by ELISA. Mean values and standard deviations of three independent analysis are represented. A reaction buffer (B) was used as a blank control.

pBI 121constructs and a non-transgenic control were probed. A VHH transcript was found in the four lines of transgenic tobacco (nos. 2, 6, 7 and 12), and the mRNA levels in individual transgenic lines were all quite different (Figure 4). A high level of a VHH transcript was found in the 6 and 7 transgenic lines. The signal was variable among different transformants, due to random integration of the foreign gene into the host tobacco genome. The size of VHH specific transcript was confirmed by RNA Century^{TM} Size Markers (Ambion, Inc., Austin, TX USA) and supplied manuals. Five micrograms of RNA Century Markers was electrophored along with RNA transcripts from transformants and cutted for staining with EtBr. The transcripts from the pBI 121 transformants were ≈ 327 bp in length by comparison with stained RNA Century Markers, which is consistent with the expected size.

Detection of VHH protein expression in transgenic plants

Based on northern analysis, the transformed plants were further checked for recombinant protein expression by ELISA analysis using a monoclonal specific antibody. As a result, the expression of recombinant VHH protein was confirmed by an ELISA assay. The results demonstrated that concentrated extracts from all selected plants were positive by ELISA (Figure 5). Expression level of VHH varied significantly among the four transformed plants. Plant derived protein in the transformed plants ranged from 0.9 to 2.5 ng/50 µg of total soluble protein. Transgenic plant line 6 showed the highest level of expression, accounting for 0.005% of the total soluble protein.

DISCUSSION

Expression of the first recombinant antibody in tobacco plant opened 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., 1989). Expression of antibodies in plants is being studied for their potential uses in biotechnology. Camelidae are known to produce immunoglobulin (Igs) devoid of light chains and constant heavy-chain domains (CH1). Antigen-specific fragments of these heavy-chains IgGs (VHH) are of great interest in biotechnology applications (Muyldermans et al., 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. Selecting the most appropriate vector for use in plant transformation can be a complex task and various factors need to be considered. Here, we have used pBI-VHH plasmid which carries origins of replication from spa and colE1 to facilitate maintenance in *Agrobacterium* and *E. coli*, respectively. The presence of unique multiple cloning sites (MCSs) for introducing target genes into the T-DNA region and a bacterially expressed marker gene (kanamaycin), permits selection and maintenance of the vector in *E. coli* and *Agrobacterium*. To facilitate immuno-histochemical, biochemical and bioassay investigation,

large amount of VHH is required; therefore, we used the CaMV 35S promoter which is a very strong constitutive promoter, causing high levels of gene expression in dicot plants. Prior to that, we used the T/A cloning vector (PTZ57R) to take advantage of inserted MCS (multi cloning site) which is beneficial for cloning. M13 primers around the MCS that facilitate the gene sequencing were also used. The 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 bioreactor for human proteins from the standpoint of containment.

This study established an efficient protocol for plant regeneration and the *Agrobacterium* mediated transformation of tobacco. The effects of different *Agrobacterium* strains C58GV3101 and LBA 4404 on transformation rate of tobacco was investigated. The results indicate that the presence of pBI-VHH plasmid with kanamycin resistance in C58GV3101 increased the level of T-DNA transfer into the tobacco cells. This may be due to the higher sensitivity of C58GV3101 when compared to LBA4404. Therefore, using this *Agrobacterium* strain may provide a better opportunity to use tobacco for genetic engineering. 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. 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. The transgene copy number as estimated by Southern hybridization ranged from one to two copies (Figure 3). According to the same experiment (Olhoft et al., 2001), we can conclude that multiple integrations of the T-DNA are due to the nature of the binary plasmid. Usually, the correlation between transcript level and T-DNA copy number is known in the transgenic plant. However, we guess that differences in the amount of specific transcript for foreign gene could be due to the promoter used or to a position effect, rather than to the number of transgene copies. One of the most commonly used methods to estimate the expression level of the recombinant antibody produced in transgenic plants is the enzyme-linked immunosorbent assay. In this work, we reported the validation of an enzyme (alkaline phosphatase)-linked immunosorbent assay to determine a plant-derived antibody directed against the MUC1 concentrations up to 2.5 ng//50 µg of total protein. The binding between MUC1 synthetic peptide and the plant-derived antibody in this assay was not affected by the impurities of the samples, demonstrating that our assay is free from interference in the range of concentrations. This VHH appears to be an intrinsically stable molecule, able to accumulate in the plant cell cytosol and to maintain its functionality. At present, the expression levels of a foreign antibody in transformed plants have been shown in relatively small amounts (0.01 to 0.1% of the total soluble protein). The highest expression level for the cholera toxin B subunit in transgenic tobacco chloroplasts, up to 4.1% of the total soluble protein has been reported (Daniell et al., 2001). The use of different promoters (Arakawa et al., 2001; Chong et al., 1997), the use of plant-derived leader sequences and signal peptides (Kong et al., 2001) and mainly the modification of codon usage of the foreign protein (Mason et al., 1980) could improve expression levels in plants. More studies are required to use the different subcloning procedures by different plant vectors, and 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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