Cloning and expression of recombinant camelid single-domain antibody in Tobacco

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Abstract
Antibodies provide a suitable tool in fundamental research and their high affinity and specificity make them invaluable for diagnostic and therapeutic applications. A promising alternative to conventional antibodies are the heavy chain antibodies (VHH) of Camelidae having short length, high solubility and stability are preferred to other antibody derivatives. In this study, our goal was production of recombinant VHH antibody fragments (against cancer associated mucin, MUC1) in tobacco plants. The *VHH* gene cDNA was cloned in TA vector and then subcloned into a plant expression binary vector pBI 121. The *VHH* gene was inserted into the plant genome by agrobacterium-mediated transformation. The presence of *VHH* gene in transformed plants was confirmed by PCR. Western blot analysis showed that the recombinant VHH protein was expressed in tobacco plant. ELISA results with MUC1 antigen confirmed that the biological activity and antigen-specific responses of the plant derived VHH protein compare favorably with that of the parent recombinant antibodies. This is the first report of production of camelied VHH antibody against tumor specific antigen from two-humped camel (*Camelus bactrianus*) in plants.
Keyword: VHH Antibody; Camelidae; Tobacco.

INTRODUCTION

Antibodies have been used as diagnostic or *in vivo* therapeutic agents for the last two decades. Because of some particular characteristics, e.g. high specificity for tumor antigens and low cross-reactivity with normal cells, they have been specifically studied in cancer therapy (Galeffi *et al*., 2005). The biochemical, technical and economical limitations, as well as, the growing clinical demand for complex therapeutic proteins and also bioreactor capacity limitation, substantial interest in developing new expression systems for production of heterologous proteins have been created. Recently, the combination of antibody and plant engineering, two rapidly advancing technologies, has resulted in the expression of a diversity of molecular forms in different plant species (Fischer and Emans, 2000; Fischer and Schillberg, 2004). The capacity of plants for low-cost production of high-quality and biologically active mammalian proteins is largely documented (Twyman *et al*., 2003).

Promising alternative to conventional antibodies are the heavy-chain antibodies of the Camelidae, which do not possess light chains and CH1 domains (Hamers-Casterman *et al*., 1993) and their heavy-chain variable domains (VHH) have been proposed as valuable potential tools for biotechnology (Muyldermans, 2001). The size of these VHH fragments is reduced to a bare minimum (a single immunoglobulin domain) and their levels of expression and solubility are significantly higher than those of classical Fab or Fvs, the smallest antibody fragments (Spinelli *et al*., 2000).

MUC1 is a transmembrane molecule that the major extracellular domain is composed of tandem repeat units of 20 amino acids. There is considerable potential in MUC1 protein to be used as a tumor-associated antigen and as a possible target antigen for immunotherapy of breast and ovarian cancer (Taylor-Papadimitriou *et al*., 1999). Historically, *Escherichia coil*-based systems have been employed for anti-MUC1 scFv production (Sakurai *et al*., 1999; Asano *et al*., 2000). Referring to the above possible applications of low molecular weight antigen binder, like camels' heavy-chain antibodies, VHH antibodies may open a new prospective of accelerated, sensitive and very stable test system and targeting vehicles. Rahbarizadeh *et al*. (2004) reported the first example of a successfully

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raised heavy-chain antibodies in two-humped camel (*Camelus bactrianus*) against MUC1 peptide. Also they expressed VHH in *Escherichia coil* (Rahbarizadeh *et al*., 2005) and *Pichia pastoris* (Rahbarizadeh *et al*., 2006). In general, the microbial systems were not successful in production of high quantities of antibodies and for targeting experiments as well as efficient immunotherapy one would require high production of the functional active VHH protein.

In this research, we report construction of anti-MUC1 VHH antibody fragment and successful expression of biologically active form of the VHH antibody in tobacco plants. This is the first report of production of camelid VHH antibody from *Camelus bactrianus* in plant.

MATERIALS AND METHODS

Bacterial strains and vector construction: Construction and screening of anti-MUC1 VHH libraries have been described in detail elsewhere (Rahbarizadeh *et al*., 2004). Briefly, old camels were immunized with either synthetic peptides corresponding to immunodominant tandem repeat region of MUC1 mucin or with homogenized human cancerous tissues. The related cDNA was synthesized and cloned in pCANTAB 5E phagemid. In this work, the camelid recombinant VHH antibody (against MUC1 as a tumor specific antigen) with GeneBank accession no. AY845432.1 (that a partial sequence was reported) was cloned in TA vector and subcloned in plant expression binary vector pBI 121 as follows: The *VHH* gene cDNA was amplified by PCR using two specific primers, 5′-TCTAGAGGATCCTAAACAATGGTCCTGCTACAGTCA-3′ (forward primer) and 5′-GGAAATTCGAGCTCTTAGTGAGATGGTGAC-3′ (backward primer), with *Bam*HI and *Sac*I sites (underlined) at the 5′ and 3′ ends, and the pCANTAB 5E phagemid was used as the template.

The sequence between *Bam*HI site and start codon in the forward primer was designed with regard to consensus sequence for initiation of translation in plants (Lutcke *et al*., 1987; Joshi, 1987). The amplified fragment was cloned into plasmid pTZ57R (Fermentas Ins TA clone) as a TA easy cloning vector, generating the recombinant TA-VHH, which was identified by PCR amplification, restriction enzyme digestion and sequencing. The *VHH* gene was released from TA-VHH and subcloned in binary vector pBI 121 in place of *gusA* gene under the control of *CaMV* 35S promoter. *E. coli* DH5α was used for transformation of the DNA construct. The resulting recombinant plasmid named pBI-VHH, and were check for the presence of *VHH* gene by endonuclease digestion and PCR amplification.

Agrobacterium transformation: The freeze-thaw method (Holsters *et al*., 1978) was used for mobilization of recombinant binary vector pBI-VHH into *Agrobacterium tumefaciens* strains LBA4404. The transformed cells were selected at 28ºC on Luria Bertani medium agar plates supplemented with 50 mgl^{-1} kanamycin and 100 mgl^{-1} streptomycin and checked by PCR analysis.

Genetic transformation of tobacco plants: Transformation of tobacco (*Nicotiana tabacum* cultivar *Xanthi*) with *Agrobacterium* harboring pBI-VHH was performed using the leaf disc method (Horsch *et al*., 1986). The transformants were selected on regeneration medium containing 100 mgl^{-1} kanamycin.

Molecular analysis of putative transformants by PCR: The presence of the transgene in transformed tobacco plants was confirmed by PCR amplification of the *VHH* gene using VHH specific primers. Plant genomic DNA was extracted from leaves of putative transformants and non-transformed tobacco plants by the CTAB (cetyl trimethyl ammonium bromide) method as described by Murray and Thompson (1980). The PCR was performed in a thermal cycler using following conditions: initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, synthesis at 72°C for 50 s and final extension for 10 min at 72°C. Amplified DNA fragments were analyzed by electrophoresis on 1% agarose gel.

Analysis of protein expression: Expression of the recombinant protein in the transgenic plants was evaluated by western blot analysis using monospecific polyclonal rabbit antiserum specific for camel antibodies, which also cross-reacts with VHH antibody fragments. Total soluble proteins were extracted from leaf tissues from transformed and nontransformed tobacco plants by grinding to a fine powder in liquid nitrogen and resuspended in 2 ml of extraction buffer (Tris-HCl 1M, Na_2-EDTA 1M, and 0.04% v/v 2-mercaptoetanol) per gram of leaf material. The insoluble debris were removed by centrifugation at 20,000 ×*g* for 20 min at 4°C, and the supernatant was used for expression analysis. The total soluble protein (TSP) concentration in the supernatant was estimated by Bradford protein assay (Bradford, 1976) using bovine serum albumin as a standard.

Extracted proteins were subjected to 12.5% SDS-PAGE as described by Laemmli (1970), and separated protein bands were transferred to nitrocellulose membrane. The membrane was blocked with PBS (Phosphate Buffer Saline) containing 10% (w/v) skim milk powder, washed with PBS buffer and then incubated with rabbit anti-camel polyclonal antibody conjugated with HRP (horseradish peroxidase). After washing, bands visualized with DAB (diaminobenzidine).

ELISA : The MUC1 peptide (TSA-P-1-24) were synthesized and conjugated to BSA by Q-BIOGENE Inc. Microtiter plates were coated with 1 $\mu g ml^{-1}$ of peptide-BSA and kept at 4°C overnight. The same concentration of BSA was used as a negative control. The plates were washed and blocked with a 1% solution of BSA in PBS for 1h in 37°C. After blocking, wells were washed and extracted protein of transgenic plants and non transgenic plants (as non-specific binding, NSB) were added. The wells were incubated for 2h, washed, combined with rabbit anti-camel conjugated to HRP and incubated for 1h. After washing, the enzyme activity was detected by adding 50 µl of 3, 3′, 5, 5′-tetramethyl benzidine (TMB) and H_2O_2 solution as substrate. The enzyme reaction was stopped by 50 µl of a 2N HCl and read in an ELISA reader at 450 nm.

Biological Reactivity of antibody towards different concentrations of antigen: The MUC1 synthetic peptide-BSA with different concentration (0-1 $\mu g well^{-1}$) was coated onto the wells of microtiter plates at 37°C overnight. The same concentration of BSA was used as a negative control. The plates were washed and blocked with a 2% solution of BSA in PBS for 1h at 37°C. At the end of incubation time, wells were washed and extracted proteins from the best transgenic plants (that showed the best specificity in ELISA) added to each well and the extracted from non-transgenic plant was used as NSB. The rest of the experiment was performed as explained in ELISA section.

Reactivity of different concentrations of antibody against MUC1 antigen: The peptide was coated onto the wells of microtiter plates (1 $\mu g well^{-1}$) at 37°C overnight. The same concentration of BSA was used as negative control. The contents of the wells were empted, washed, and blocked with a 1% solution of BSA in PBS for 1h at 37°C. At the end of incubation time, wells were washed and different dilutions of transgenic plant homogenized including single-domain antibody (sdAb) were added and the extracted from non-transgenic plant was used as NSB. The rest of the experiment was performed as described.

RESULTS

Construction of vectors: *TA-VHH:* PCR-amplified VHH gene (380 bp) was cloned into T/A vector, and the generated recombinant TA-VHH was identified by digestion and PCR amplification (Fig. 1). Also, the results of sequencing showed that the fragment was amplified and cloned without any mutation. *pBI121-VHH:* The VHH fragment excised from TA-VHH was cloned into pBI121, and the recombinant pBI-VHH was confirmed by digestion (Fig. 2). In this construct, the antibody gene was located between the cauliflower mosaic virus (CaMV) 35S promoter and nopaline synthase terminator. After confirmation of pBI-VHH, the

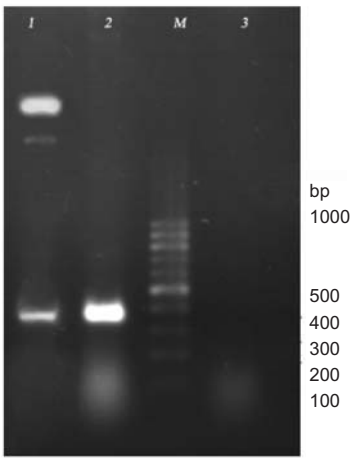

Figure 1. Identification of recombinant vector T/A-VHH. 1, digestion of T/A-VHH with *Bam*HI + *Sac*I that released a 380 bp fragment; 2, PCR amplification of VHH gene using T/A-VHH as template; 3, negative control for PCR amplification; M, 100 bp DNA size marker.

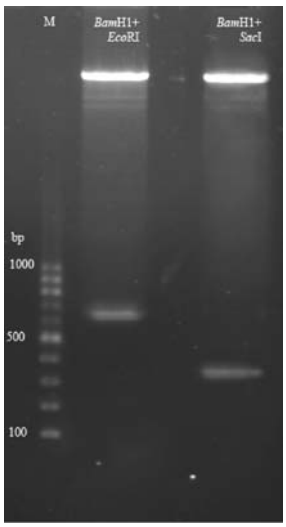

Figure 2. Identification of recombinant vector pBI-VHH. M: 100 bp DNA size marker, digestion of pBI-VHH with *Bam*HI + *Sac*I released about 380 bp fragment and with *Bam*HI + *Eco*RI released about 680 bp fragment.

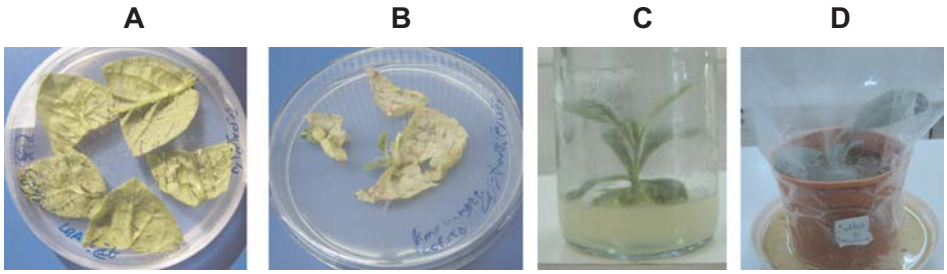

Figure 3. Different regeneration stages of the transgenic tobacco plants. A: transformed leaves on regeneration medium containing antibiotics, B: 3-4 week leaf tissues showing regeneration of adventitious shoot, C: elongated rooted shoot on root induction medium containing kanamycin, D: transformed plants in soil.

recombinant vector was mobilized into *Agrobacterium tumefaciens* strains LBA4404 and the transformed cells were checked by PCR analysis.

Transformation and regeneration of tobacco plants: Genetic transformation of tobacco was efficient and resulted in enough numbers of transgenic plants. Shoot regeneration occurred via adventitious proliferation from leaf tissues on selection medium after 2-3 weeks (Fig. 3A, B). Elongated shoots measuring 4-6 cm in length were subcultured to root induction medium containing 100 mgl^{-1} kanamycin (Fig. 3C). Shoots which apparently escaped from previous selection eventually died within 2-3 weeks on root induction medium and only those shoots that were transgenic rooted on the selection medium and the escapes mostly did not produce root. Two to three weeks after rooting, plantlets were transferred to vermiculite soil (Fig. 3D) and placed in the temperature-controlled (25-30°C) greenhouse. All transformants appeared morphologically normal in comparison with non-transformed plants.

Confirmation of transformats by PCR analysis: PCR amplification was performed to analyse the presence of the recombinant *VHH* gene in transformed plants, using plant DNA extracted from putative transformed tobacco lines and VHH specific primers. A dsDNA of 380 bp was amplified from the transgenic lines (Fig. 4). No VHH PCR products were seen for untransformed control plants. Based on the kanamycin and PCR amplification, selected transgenic lines were used for Western blotting and ELISA analysis.

Analysis of antibody expression by Western blotting: The PCR positive lines were further tested for the detection of VHH protein by Western blot analysis, using rabbit anti-camel polyclonal antibody. Proteins extracted from the putative transgenic tobacco were separated on 12.5% SDS-PAGE gel. A protein band of about 15 kDa was detected on immunoblots, confirming the expression of the VHH protein (Fig. 5). The expression of VHH protein was detected in seven plants (L1, L2, L3, L5, L7, L8 and L11).

ELISA analysis: The specifity of soluble single domain antibodies towards the antigen was determined by ELISA. Significant differences between optical densities (ODs) at 450 nm of transgenic plant proteins against MUC1-related peptides and BSA (negative control) indicated that plant-derived antibodies from seven transgenic plants successfully reacted against the MUC1-related peptides (Table 1). However, this antibody did not show any cross-reactivity toward non-specific proteins from wild type plant.

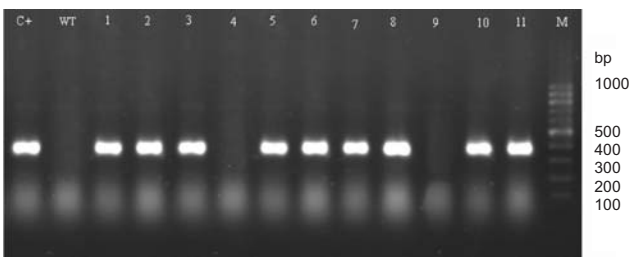

Figure 4. Electrophoresis of the PCR products of putative transgenic tobacco plants. M: 100 bp DNA size marker, C+: positive control, WT: untransformed tobacco plant, 1-11: transformed tobacco plants.

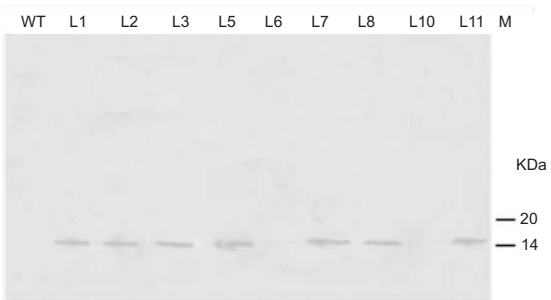

Figure 5. Western blot analysis of VHH expression in transgenic tobacco plants. M: protein molecular weight marker, WT: untransformed tobacco plant (negative control), L1, L2, L3, L5, L6, L7, L8, L10, and L11: transgenic tobacco plants.

Table 1. ELISA results of transgenic plant proteins containing VHH.

Antigen	Transgenic plant							Non-transgenic
	L 1	L 2	L 3	L 5	L 7	L 8	L 11	
BSA	0.156	0.143	0.235	0.160	0.201	0.128	0.152	0.216
MUC1 peptide- BSA	0.583	0.570	0.491	0.750	0.862	0.474	0.552	0.221

All assays were performed in duplicate and results are the average of binding activity of VHH towards synthetic peptide antigen. L1, L2, L3, L5, L7, L8 and L11 are transgenic tobacco plants and non-transgenic plant that was used as the NSB.

Specific activity of VHH towards different concentrations of MUC1 peptide: The biological reactivity of recombinant single domain antibody from the transgenic lines 5 and 7 which showed the best specificity in ELISA with different concentrations of synthetic peptide conjugated to BSA was tested using ELISA (Fig. 6). The recombinant antibody showed high immunoreactivity towards MUC1-related peptide, and almost no cross-reaction was shown towards non-specific proteins from non-transgenic plant.

Reactivity of sdAb against antigen: Each antibody preparation was evaluated for immunoreactivity by titration in ELISA with MUC1 peptide. The resulted antibodies of both transgenic plants (L5 and L7) at different concentrations showed high immunoreactivity (Fig. 7).

DISCUSSION

Immunoglobulins constitute at least 25% of the proteins in clinical trails and these molecules are almost exclusively applied in human therapy and diagnosis. For most applications, high-yield production, solubility, stability, and small size are critical factors. However, many attempts to reduce the size of antibody such as Fvs, scFv and even single domain VH, while retaining antigen-binding properties, have been reported. Antigen-specific fragments of camelid heavy-chain IgGs (VHH) are of great interest in biotechnology applications (Muyldermans *et al*., 2001). Although recombinant VHH against MUC1-related peptide were expressed in *Escherichia coil* (Rahbarizadeh *et al*., 2005) and *Pichia pastoris* (Rahbarizadeh *et al*., 2006), the microbial expression system have many limitations. Several reports have demonstrated that plants can produce full-size antibodies and antibody fragments and potentially are the most economical production system for functional recombinant antibodies.

There are some reports on the production of many different antibody forms against tumor-associated antigens including full-length immunoglobulin and artificially constructed derivatives in plants (Ko *et al*., 2005; Khoudi *et al*., 1999; Mallender *et al*., 1996; Porceddu *et al*., 1999; Smith and Glick, 2003; Ma *et al*., 2003). Also, Rajabi-Memari *et al.* (2006) recently reported the first example of VHH antibody from one-humped camel (*Camelus dromedarius*) against tumor-associated marker in tobacco. However, there is no report of production on VHH antibody fragments from two-humped camel (*Camelus bactrianus*) in plants. In

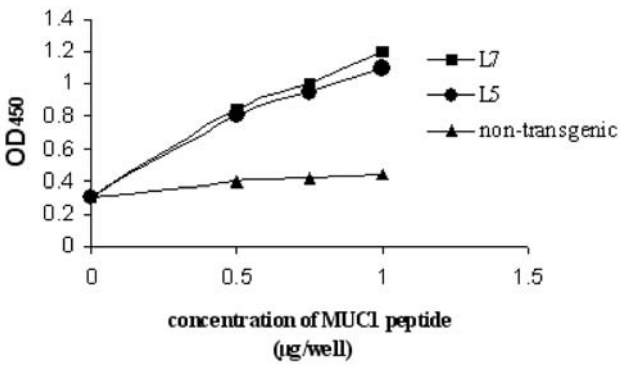

Figure 6. Western blot analysis of VHH expression in transgenic tobacco plants. M: protein molecular weight marker, WT: untransformed tobacco plant (negative control), L1, L2, L3, L5, L6, L7, L8, L10, and L11: transgenic tobacco plants.

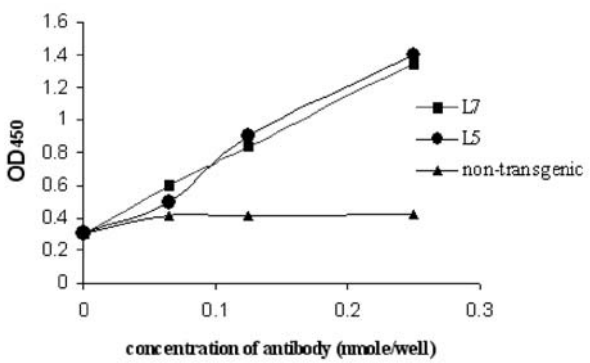

Figure 7. Reactivity of single-domain antibody obtained from transgenic line 5 and 7 towards different concentrations of MUC1 peptide (TSA-P1-24-BSA). NSB was determined using non-transgenic plant.

this work, we produced the recombinant VHH antibody against tumor-associated marker (MUC1) in tobacco plants and data presented suggested that plant system is a suitable host for expression of a biological active form of VHH antibody.

In the present study, expression of recombinant antibody in transgenic plants was confirmed by Western blot analysis. However in some batches of transformed plants, although *VHH* gene was detected by PCR analysis, the protein expression was not seen. This may be due to a low level expression of recombinant protein below the detection threshold of antibody by a recombinant event within T-DNA, which has altered *VHH* gene structure (Prasad *et al*., 2003).

Various approaches and considerations have been suggested to increase the target protein expression level in transgenic plants, including type of promoter, introducing of a 5′-untranslated leader (5′UTL) sequence and start codon context. The most commonly used constitutive promoters are of viral origin such as CaMV 35S promoter that derives very high levels of transcription in most tissues of the plant (Benfey *et al*., 1990; Lessard *et al*., 2002). As has been demonstrated in mammalian systems (Kozak, 1992), the context sequence of the start codon in plants can have a strong effect on the level of expression of a transgene, although the specific context differs between plants and animals (Lutcke *et al*., 1987; Joshi, 1987; Taylor *et al*., 1987). Recently, it has been demonstrated that conserved nucleotides downstream of the start codon could augment post-transcriptional effects on gene expression (Sawant *et al*., 2001). In this work, we considered the consensus sequence for initiation of translation in plants downstream of the start codon in the forward primer. Other consideration such as strong promoter and terminator were available on universal plant expression vector pBI 121. Our data on the expression VHH antibody in transgenic plants suggest that this system and constructed plasmid can potentially be used for antibody production.

The biological activity and antigen-specific responses of the plant-derived VHH with MUC1 peptide was examined by ELISA and the results confirmed that the plant derived recombinant VHH can successfully react against the MUC1-related peptide. Also, the immunological activity of the plant derived VHH was compared with that of the parent recombinant VHH antibodies reported by Rahbarizadeh *et al.* (2004) and we found that the activity of plant derived antibodies compare favorably with that of the original VHH antibody. The results presented here demonstrated that the tobacco plants would be the choice for production of anti-MUC1 VHH antibodies and as an alternative to hybridoma or microbial production systems. More work is necessary in order to optimize antibody production in plants and the extraction/purification protocols that have a substantial influence on final yields, with respect to medical use.

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