

# Expression of anti-K99 scFv in transgenic rice tissues and its functional characterization

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**Abstract** As a first step towards manufacturing functional anti-K99 single chain variable antibody fragment (scFv) in a plant system to prevent colibacillosis in neonatal calves, we investigated the feasibility of producing these antibodies in rice plants. Two scFv constructs, with or without the endoplasmic reticulum (ER) targeting KDEL sequence, were introduced into rice for either ER-retention of the recombinant antibody or its secretion. In agreement with several other published reports, extremely low-levels of scFv were produced in rice plants transformed with the construct lacking the ER-targeting sequence. Constructs containing the KDEL sequence resulted in significantly higher levels of the antibody in rice leaves. Although scFv transcripts were found in all three rice tissues analyzed, scFv protein was detected only in the leaf and embryo tissues and not in the endosperm portion of the seed. Functionality of the rice-produced scFv was tested in two in vitro assays, i.e., inhibition of K99-induced

horse red blood cell agglutination and inhibition of the attachment of enterotoxigenic *Escherichia coli* (ETEC) to calf enterocytes. Rice-scFv was found to be functionally equivalent to anti-K99 monoclonal antibody (mAb) in both the assays. The results obtained in this investigation provide valuable information and in combination with other studies of this kind, will be helpful in devising strategies to improve production of useful recombinant proteins in the seeds.

**Keywords** ETEC · K99 · KDEL · Rice · scFv · Transgenic

## Introduction

Protein-based therapeutics produced by bacterial, yeast or mammalian cells are finding increasing usage in treating various human diseases and medical conditions. The market size for these at ~\$33 billion in 2004 is estimated to grow to \$70 billion by the end of this decade (Walsh 2006). Although highly effective, such cell culture-produced therapeutic molecules are too expensive for use in large-scale, veterinary applications. Antibody production systems based on the use of whole animals are also cost-prohibitive for passive immunization to treat veterinary diseases. Recombinant plants offer practical

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and economic alternative for prophylaxis in domestic animals. In addition, certain recombinant plants can be ideal production systems when the preferred mode of delivery of the biopharmaceutical protein is through the oral route.

Calf scours or diarrhea is one of the leading causes of financial loss to the dairy and beef industry. Enteric colibacillosis, a major scour disease, is caused by a noninvasive enterotoxigenic *E. coli* (ETEC) in neonatal calves, piglets and lambs (Gaastra and de Graaf 1982). Following oral infection ETEC colonize the mucosal surface of the small intestine and secrete enterotoxins. Watery diarrhea induced by the action of these toxins leads to severe fluid loss and dehydration which may result in the death of the animal. The pathogenesis starts with the attachment of the ETEC to the brush border of the epithelial cells in the intestine. The attachment to the ganglioside receptors on the enterocytes is mediated by binding motifs on long, thread-like, proteinaceous pili or fimbriae designated K99 (F5) (Burrows et al. 1976; de Graaf et al. 1981), common to most ETEC strains affecting calves (Acres 1985; Moon and Bunn 1993). Since the binding of ETEC to the enterocytes is an early and critical step in the disease development, blocking this attachment serves as an effective strategy to protect young calves from colibacillosis.

There is ample evidence to show the protective ability of passively administered anti-K99 antibodies. Supplementary feeding of calves with colostrum from cows vaccinated with purified K99 or other forms of K99 such as whole cell bacterins or crude cell preparations provide protection against ETEC infection through passive immunity (Acres et al. 1979; Nagy 1980; Snodgrass et al. 1982). The whole immunoglobulin molecule is not necessary since binding of ETEC to the specific receptors is also prevented by Fab fragments of the anti-K88 or anti-K99 antibody (Isaacson et al. 1978; Sun et al. 2000). Oral administration of a monoclonal antibody (mAb) to an epitope on K99 pili significantly reduces neonatal calf mortality (Morter 1984; Sherman et al. 1983). In the above-mentioned studies, the effectiveness of anti-K99 antibodies depends on their binding to the K99 antigen and thereby inhibiting the attachment of ETEC to the receptors on epithelial cells. Such inhibition of attachment prevents multiplication of ETEC and therefore development of the disease.

Current practice to reduce calf scours is vaccination of pregnant dams with antigen prior to calving [i.e., ScourGuard 3<sup>®</sup> (Pfizer), Scour Bos<sup>®</sup> 9 (Novartis)] or supplementary administration of antibodies to calves [Bovine Ecolizer<sup>®</sup> (Novartis), Bar-Guard-99<sup>™</sup> (Boehringer Ingelheim), First Defense<sup>®</sup> (ImmuCell)]. However, the vaccination procedure requires that a cow must receive multiple immunizations to attain a high titre of antibodies in the colostrum (Haggard et al. 1982). Although the vaccination may induce high levels of K99-specific antibodies in the colostrum, the calves may fail to suckle during the critical initial period, leading to failure in the effectiveness of the vaccine. Commercial antibody preparations are costly and require the use of animals or mammalian cell cultures for the production. Transgenic plants provide an alternative system for the production of therapeutic compounds that is significantly less expensive. Use of transgenic plants for the production of functional therapeutic compounds has been well documented during the past two decades. In addition to being more economical, plant systems offer several other benefits. These include a non-animal system for large-scale production, avoidance of potential contamination with pathogens or bacterial endotoxins, and requirement of minimal protein purification steps as many plant products can be consumed raw or as crude preparations. Casadevall (2002) lists some of the biowar agents against which specific antibodies can be effective and suggests stockpiling antibody-based reagents for immediate prophylaxis of susceptible populations. Antibodies manufactured in seeds would be a good source of a reagent that can be stored economically.

Following the demonstration of successful production and assembly of functional antibodies in transgenic tobacco by Hiatt et al. (1989), several forms of antibodies such as full-length immunoglobulins or its derivatives like single chain variable fragments (scFv), Fab fragments or heavy-chain variable domains have been expressed in plants (Daniell et al. 2001; Floss et al. 2007). The scFvs are potential alternatives to the full-length antibodies as they are smaller in size and retain the essential binding specificity of mAbs. The scFvs are usually expressed from constructs encoding functional domains of the light and heavy chains of the antibody that are linked by a short nucleotide sequence encoding a flexible polypeptide linker (Bird et al.

1988). A construct encoding anti-K99 scFv had previously been assembled in our laboratory from a hybridoma cell line producing mAb against K99 (Bhaskaran et al. 2005). The recombinant anti-K99 scFv produced in *E. coli* maintained the ability to inhibit agglutination of horse red blood cells (HRBCs) induced by K99 antigen (HRBC has the same ganglioside receptor as the enterocyte). Moreover, this scFv was able to prevent the attachment of ETEC to calf enterocytes, thus suggesting that anti-K99 scFv can serve as an effective therapeutic agent to control colibacillosis. Production of the scFv in plants and supplemental feeding of neonatal calves with this scFv can be potentially a more cost-efficient and practical solution to control enteric colibacillosis compared to the use of more expensive mAbs.

In this study, while characterizing scFv against K99 antigen produced in transgenic rice under the control of CaMV 35S promoter, important and interesting differences amongst leaf, embryo, and endosperm tissues pertaining to scFv accumulation were revealed. We demonstrate that the recombinant scFv had the ability to inhibit the adherence of K99 antigen to the receptors on HRBCs. More importantly, the binding ability of ETEC to the calf intestinal enterocytes is significantly hampered by rice-produced scFv.

## Materials and methods

### Construction of scFv expression vectors and rice transformation

The anti-K99 scFv gene was originally constructed from the cDNA prepared from a hybridoma cell line expressing anti-K99 mAb (Bhaskaran et al. 2005). The scFv gene was assembled by linking the PCR amplified DNA fragments encoding the light and heavy chains with an oligonucleotide encoding a peptide linker (Gly<sub>4</sub>Ser)<sub>3</sub> and introduced into pCANTAB5. This scFv gene in the resulting *E. coli* expression vector pSHOB-52 (Bhaskaran et al. 2005) was used to construct the plant expression cassette used in this study. Following primers were used to isolate the scFv gene along with the 3' end-located E-tag from pSHOB-52: scFv-F: 5'-CCACAATATTATGGCCCA GGTC AAGCTGCAGCAG-3' and scFv-R1: 5'-CGT

AGATCTCTAagactcatcctTGCGGCACGCGGTTCC AGCGGA-3'. The sequence encoding the ER-targeting signal KDEL incorporated into the reverse primer is indicated in lowercase. Another version of the scFv gene, that lacked the sequence for KDEL signal, was amplified to assemble a second construct for comparison purpose by using the forward primer scFv-F in combination with a different reverse primer, scFv-R2: 5'-CGTAGATCTCTATGCGGCACGCGGTTCCAG CGGA-3'. The PR1 secretory peptide signal sequence was isolated from *Nicotiana tabacum* genomic DNA by PCR using the primers PR1-SP-F: 5'-CCC GC CATGGGATTTGTTCTCTTTTCAC-3' and PR1-SP-R: 5'-AGTCCCGGGTTGTTGAGAGTTTTGGGAC GG-3'. The PR1 signal peptide and the scFv gene with or without KDEL were ligated into the binary vector pRTL2 (Restrepo et al. 1990) in a three-way ligation to create pRTL-K99-scFv-SK and pRTL-K99-scFv-S. The plant expression cassette containing a CaMV 35S promoter with a double enhancer, a translational enhancer from tobacco etch virus (TEV), scFv gene and the terminator from each of the pRTL-based scFv constructs were isolated and ligated into pCAM-BIA1300. The resulting plant transformation vectors K99-scFv-SK (with KDEL) and K99-scFv-S (without KDEL) were transferred to *Agrobacterium tumefaciens* strain EHA105. Callus cultures derived from mature seeds (japonica rice cv. Taipei 309) were transformed and the transgenic plants were regenerated as previously described (Aldemita and Hodges 1996). The transgenic plants were maintained in the greenhouse until maturity.

### Extraction and purification of recombinant scFv

Twenty whole mature rice seeds or endosperms were ground to fine powder using mortar and pestle. The powder was further homogenized in 3 ml (for Western blot) or 1.2 ml (for ELISA) of PBS (0.1 M phosphate buffer, 0.15 M NaCl, pH 7.2) containing 1% Triton X-100. Twenty embryos isolated from mature seeds, 14 days post anthesis (dpa) or 21 dpa seeds were homogenized in a microfuge tube with pellet pestle in 600 µl of PBS containing 1% Triton X-100. In the case of leaves, ~200 mg of 3 mm long segments of freshly opened leaf samples were homogenized with 20 mg of sand in 1 ml of PBS buffer using mortar and pestle. The homogenate was centrifuged at 13,000 rpm for 15 min at 4°C. The

homogenate or the supernatant containing total soluble proteins (TSP) was used for ELISA and Western blot analyses. The anti-K99 scFv from the supernatant fraction was purified using Protein-L affinity column (Pierce, Cat #20520) by following the protocol recommended by the manufacturer. The bound scFv antibodies were eluted with 0.1 M glycine–HCl buffer (pH 2.5) and the pH of the eluate was immediately neutralized by adding 1/10th volume of 1 M PBS buffer (pH 7.5). The quantity of the purified recombinant scFv was estimated using Western blot analysis by comparing the band intensity of rice-derived scFv with that of a known quantity of *E. coli*-derived recombinant scFv.

#### Enzyme-linked immunosorbent assay (ELISA)

Initial screening of transgenic rice plants to assess the expression of recombinant anti-K99 scFv was performed by ELISA on the leaf tissue extracts. In addition, this technique was also used to determine the scFv levels in the seed tissue. ELISA was performed as described by Jay et al. (2004) with 25 µg of TSP extracted from rice leaf tissues or 50 µl of homogenate from the seed tissue. The 13 amino acid-long peptide (E-Tag) at the carboxyl termini of the recombinant scFv allowed us to use anti-E-Tag mAb (Amersham Pharmacia) as the primary antibody for detection in ELISA at 1:500 dilution. Horseradish peroxidase-conjugated goat anti-mouse IgG (whole molecule, Sigma, Cat. # A5278) was used as the secondary antibody at 1:3,000 dilution. 2,2-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) was used to detect the presence of the bound conjugate.

#### Western blot analysis

Forty microgram of TSP extracted from rice leaf or 14 µl of seed tissue homogenate were separated on a 12.5% polyacrylamide SDS–PAGE gel (Laemmli 1970). The proteins were transferred (Towbin et al. 1979) onto PVDF membrane (Immobilon-P, Millipore, Cat# IPVH00010) using Mini Trans-blot® electrophoretic transfer cell (Bio-Rad, Cat #170-3930; 170-3935). The electrophoretic transfer was performed at 300 mA for 3 h in 25 mM Tris buffer containing 192 mM glycine and 10% methanol. After transfer, the blots were rinsed with distilled water and dried at room temperature for 4 h. Blocking, antibody

treatments and washing steps were carried out at room temperature in a solution containing 5% milk protein (Carnation Non-fat Dry Milk) in TBS buffer (10 mM Tris and 150 mM NaCl, pH 8.0). The blots were blocked for 2 h and incubated with anti-E-tag antibody as the primary antibody (1:1,000 dilution) for 2 h followed by four washes for 5 min each. The blots were then incubated with anti-mouse HRP conjugate (1:3,000 dilution) as the secondary antibody for 1 h. This was followed by four washes of 5 min each with TBS buffer containing 5% milk protein and 0.05% Tween-20. The antibody binding was detected by ECS chemiluminescence method as described below. The blots were incubated in 0.1 M Tris buffer (pH 8.9) containing 1.25 mM luminol, 200 µM *p*-coumaric acid and 0.01% hydrogen peroxide at room temperature for 2 min. After draining the excess fluid, the blots were wrapped inside a sheet protector and exposed to X-ray film for 2 min.

#### Haemagglutination inhibition

HRBCs were prepared for haemagglutination inhibition experiment by following the protocol described by Jay et al. (2004). Haemagglutination titre was performed to identify the minimum amount of K99 protein required to cause complete agglutination of the HRBCs. A 100 µl sample of 0.5% HRBC was mixed with a twofold serially diluted, purified, *E. coli*-produced K99 protein in 100 µl of PBS buffer in a 96-well round bottom microtiter plate and the plate was observed after incubation at 4°C for 4 h. Haemagglutination inhibition assay was performed as described below. Various concentrations of purified rice recombinant scFv or anti-K99 mAb were combined each with 3.1 ng of K99 protein in a final volume of 100 µl of PBS buffer and pre-incubated for 10 min at 37°C. A 100 µl sample of 0.5% HRBC suspension was mixed with this antibody/K99 mixture and incubated for 4 h at 4°C. The plates were photographed following incubation.

#### In vitro binding inhibition assay

The ETEC K99<sup>+</sup> (*E. coli* O101:K-:K99 strain B41) cells were grown to 0.6 OD at 37°C in E-medium (Bhaskaran 2002). Bovine enterocytes were harvested and fixed by following the method described by Jay et al. (2004). Six microgram of mAb or 3.1 µg of

scFv were mixed with  $5 \times 10^4$  bacterial cells in a 6 ml glass tube to a final volume of 50  $\mu$ l in PBS buffer and incubated for 30 min at 37°C on a shaker (250 rpm). The final concentrations of the mAb and scFv were 0.8 and 2  $\mu$ M, respectively. The enterocytes ( $8 \times 10^4$  cells) or HRBCs ( $1.25 \times 10^5$  cells) were mixed with the bacterial cells and incubated as above for another 30 min. Ten microliter of this cell suspension was spread on a glass slide, heat-fixed (55°C for 5 min) and stained with Giemsa stain (Sigma). The enterocytes or HRBCs with *E. coli* attached on the surface were counted at 100 $\times$  magnification under a light microscope.

### RNA isolation and RT-PCR analysis

Various tissues including 3 mm long segments of leaves, immature seeds (14 dpa), embryos (14 dpa) and endosperm (14 dpa) were stored in RNAlater (Ambion, Inc.). Ten immature embryos or 100 mg of leaf tissue were homogenized in 550  $\mu$ l of RNA isolation buffer [4 M guanidine isothiocyanate, 30 mM disodium citrate (Ambion, Inc. Cat. # 7020)] with Proteinase K (1.5 mg per sample) and 30 mM 2-Mercaptoethanol using mortar and pestle. In the case of seeds, five each of the developing seeds or endosperms were homogenized in 2 ml of RNA isolation buffer containing Proteinase K (1.5 mg per sample) and 30 mM 2-Mercaptoethanol. The total RNA was isolated from the extracts using RNeasy Plant Mini Kit (Qiagen, Cat. # 74904). Reverse-transcription reaction was carried out using Taqman Reverse Transcription Reagents kit (Applied Biosystems, Cat. # N808-0234) with 400 ng of total RNA and oligo (dT) primers in a 10  $\mu$ l volume. One microliter of the synthesized first-strand cDNA was used in a PCR to amplify scFv cDNA (amplicon size: 800 bp) using the following primers: scFv-F: 5'-CG GAATTCATGGCCAGGTCAAGCTGCA-3' and scFv-R: 5'-GCGAAGCTTTGCGGCACGCGGTTCC AGCG-3'. As an RNA quantity control, rice actin gene was amplified (amplicon size: 150 bp) using the following primers: Act-F: 5'-CTCTCAACCCCAAG GCCAA-3' and Act-R: 5'-GTACGACCACTGGCAT AC-3'. The PCR conditions were as follows for both sets of primers: 94°C for 5 min; 29 cycles of 94°C for 45 s, 60°C for 45 s, and 72°C for 90 s; and a final extension at 72°C for 10 min.

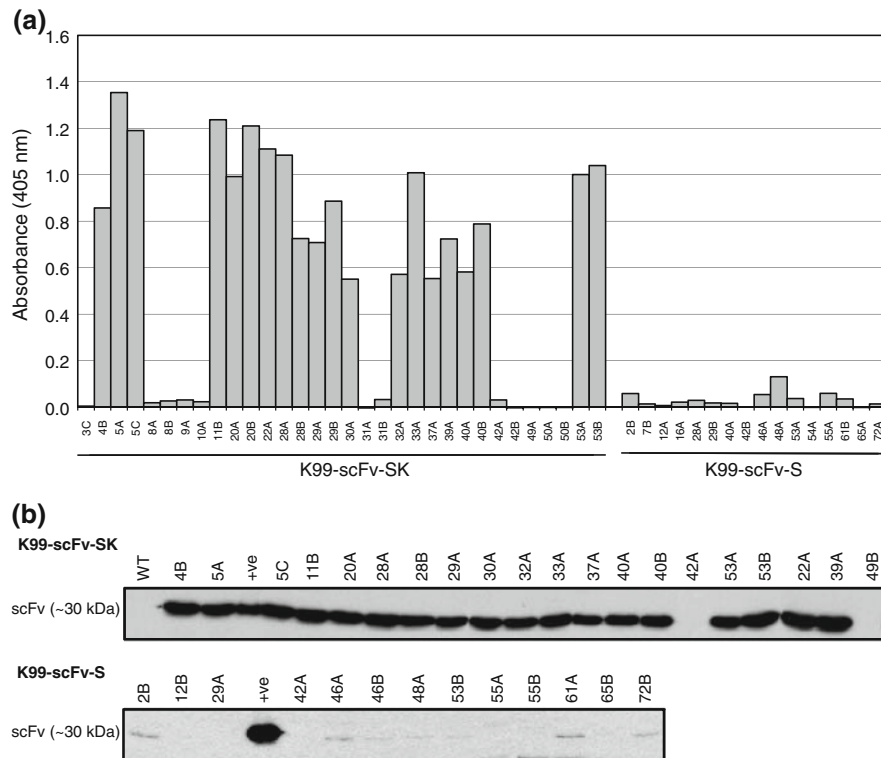
## Results

### Expression of scFv in transgenic rice leaves

A total of 32 and 16 transgenic rice plants were generated with constructs K99-scFv-SK and K99-scFv-S, respectively. Initial screening to examine the expression of the scFv in transgenic rice plants was carried out using ELISA. Leaves from about 20 putative transgenic plants transformed with the construct K99-scFv-SK showed positive ELISA reactions, indicating the presence of anti-K99 scFv protein (Fig. 1a). Leaves from only six transgenic plants, transformed with the construct K99-scFv-S, showed the presence of scFv albeit at very low levels compared to those in the K99-scFv-SK transformants. The results obtained from ELISA were further confirmed by performing Western blot analysis on protein extracts from the leaves of some of the plants transformed with each of the two constructs (Fig. 1b). Proteins from the ELISA-negative plants or from non-transgenic control plant did not react with the anti-E-tag antibody. However, total protein isolated from all of the ELISA-positive plants showed a band on Western blot of an expected size of  $\sim$ 30 kDa that reacted with the anti-E-tag antibody confirming the expression of anti-K99 scFv in these plants. The expression levels of scFv-KDEL ranged from 0.85 to 1.44  $\mu$ g per mg of total soluble leaf protein in various K99-scFv-SK transgenic lines. Seven of the K99-scFv-S transgenic plants showed scFv expression at very low, but detectable levels on the Western blot. The scFv concentrations in these plants were within a range of 0.01–0.02  $\mu$ g per mg of total soluble leaf protein. All of the scFv expressing transgenic plants had growth patterns comparable to the non-transgenic controls and were fertile.

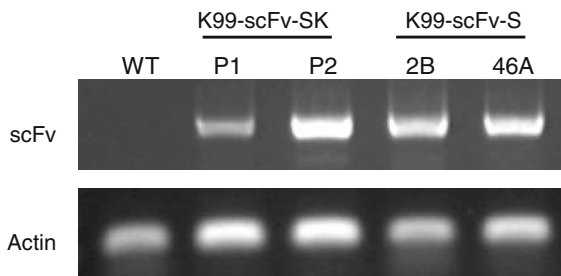
### scFv transcripts in rice leaves

In order to test whether the poor expression of scFv protein in the leaves of K99-scFv-S transformants was due the lack of the promoter activity, we carried out RT-PCR on the total RNA isolated from the leaves from the two types of transgenic plants. The results suggest that 35S promoter, which controls the transcription of the scFv gene in both K99-scFv-SK and K99-scFv-S constructs, is functional in the



**Fig. 1** ELISA and Western blot analyses of scFv expression in the leaf tissue from transgenic (T0) rice plants. **a** ELISA-based detection of scFv in the leaf tissues from 32 K99-scFv-SK transformants and 16 K99-scFv-S transformants. The bars represent values obtained after subtracting non-transgenic control leaf reading from each transformant ELISA reading. **b** Western blot analysis performed on protein samples from a

wild-type (WT) control plant and several transgenic lines transformed either with construct K99-scFv-SK or K99-scFv-S. Forty microgram of total soluble protein was loaded in each lane and anti-E-tag antibody was used for detection. The lane labeled +ve contained protein from a previously confirmed tobacco callus transformed with K99-scFv-SK



**Fig. 2** RT-PCR analysis of scFv gene expression in transgenic rice leaf tissue. Total RNA isolated from leaf tissues from wild-type (WT), two progeny plants (P1 and P2) from line K99-scFv-SK-37A, and two independent transgenic lines K99-scFv-S-2B and K99-scFv-S-46A were analyzed. Transcripts amplified from rice actin gene served as RNA quantity control

leaf tissue (Fig. 2). Thus, it is not the lack of promoter activity that can account for such low levels of the antibody in K99-scFv-S leaves.

### Expression of scFv in rice seeds

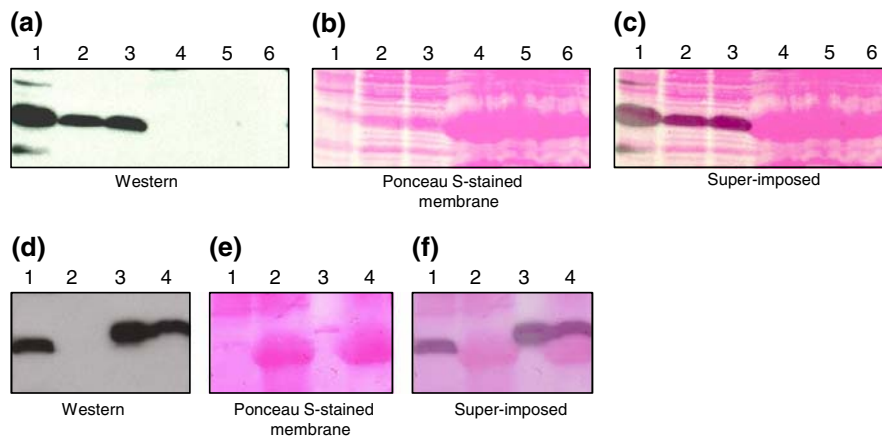
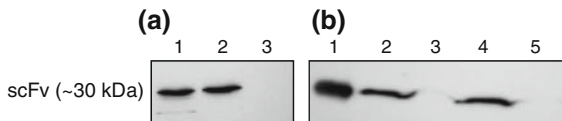
Our early attempts to detect scFv in the mature seeds of any of the K99-scFv-SK transformants by Western blot analysis were unsuccessful. However, RT-PCR analysis on 14 dpa seeds showed that the antibody gene was being transcribed (data not shown). Based on this positive result, we wondered whether or not these messages were translated into their respective proteins in the developing seeds. Therefore, we examined extracts from 14 dpa seeds, 21 dpa seeds, and mature seeds by Western blot analysis. The results from this analysis, presented in Fig. 3a, clearly show that the scFv protein is present in the developing seeds. A possible reason for the absence of detectable band on a Western blot in the case of mature seed may be the presence of certain interfering factors in the storage compartment of the seed,

the endosperm. In order to explore this possibility, we manually separated the embryo from the endosperm in 21 dpa seeds as well as mature seeds. Western blot analysis performed on the extracts from these tissues is presented in Fig. 3b. Embryos from both types of seeds showed the presence of scFv while the endosperm tissue in each case showed no detectable antibody. The fact that 21 dpa whole seed extract did show a detectable scFv band (Fig. 3a), suggests that

the interfering factor is produced some time after this phase in seed development. To further confirm that our inability to detect scFv on the Western blot was due to interference from endosperm, we conducted the following experiment. Protein extracts of either leaf tissue or the embryos from two different K99-scFv-SK transgenic lines were analyzed either by themselves or following mixing with heat-denatured extracts from non-transgenic endosperm tissue. The result from this Western blot is presented in Fig. 4a. Leaf and embryo extracts from the two lines, 37A and 22A, show the antibody band. On the other hand, the lanes loaded with the mixture (leaf + endosperm or embryo + endosperm) show no detectable band confirming that some factor present in the endosperm tissue interferes with the detection of the antibody during Western analysis.

Close observation of the PVDF membrane following staining with Ponceau-S as well as the polyacrylamide gel stained with Coomassie Brilliant Blue revealed the presence of a thick protein band of a size similar to the scFv in lanes containing endosperm extract, most probably representing

**Fig. 3** Western blot analysis of scFv expression in various tissues from transgenic rice plants. **a** scFv expression in 14 dpa seed (lane 1), 21 dpa seed (lane 2), and mature seed (lane 3) from line K99-scFv-SK-37A. Total soluble proteins were extracted from a sample of ten pooled seeds for each category. **b** scFv expression in leaf (lane 1), embryo (lane 2) and endosperm (lane 3) tissues derived from 21 dpa seeds, and embryo (lane 4) and endosperm (lane 5) tissues derived from mature seeds. Pooled samples of embryos or endosperms from 20 seeds were used for this analysis



**Fig. 4** Interference with the detection of rice-produced scFv in a Western blot by endosperm extract. **a** Immunoblot performed on total soluble protein extracts from leaf tissue from K99-scFv-SK-37A plant (lanes 1 and 4), 21 dpa embryo from K99-scFv-SK-37A plant (lanes 2 and 5), and 21 dpa embryo from K99-scFv-SK-22A plant (lanes 3 and 6) after mixing the respective sample with either an equal portion of extraction buffer (lanes 1, 2 and 3) or with an equal portion of heat-denatured extract from non-transgenic, mature seed endosperm tissue (lanes 4, 5 and 6). **b** Image of the Ponceau-S stained PVDF membrane used in the Western blot shown in **a**. **c** Superimposed images of the immunoblot (**a**) and the stained membrane (**b**) showing a thick protein band, in the lanes

containing the endosperm extracts (lanes 4, 5 and 6), of a size similar to the scFv. **d** Immunoblot performed on total soluble protein extracts from leaf tissue from K99-scFv-SK-37A plant (lanes 1 and 2) and bacterially-expressed scFv (lanes 3 and 4) after mixing the respective sample with either an equal portion of extraction buffer (lanes 1 and 3) or with an equal portion of a non-transgenic, mature seed-endosperm-extract (lanes 2 and 4). **e** Image of the Ponceau-S stained PVDF membrane used in the Western blot shown in **d**. **f** Superimposed images of the immunoblot (**d**) and the stained membrane (**e**) showing an overlap between the thick protein band and rice-scFv (lanes 1 and 2). The slightly larger bacterial-scFv does not show this overlap (lanes 3 and 4)

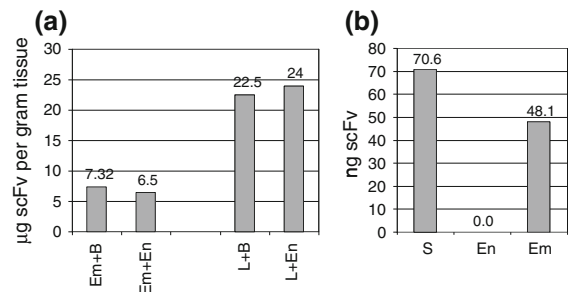
storage protein(s). This band was not seen in the lanes with extracts from leaf tissue or embryos (Fig. 4b, c). It is possible that the presence of the endosperm storage protein(s) interfere either with the transfer of the scFv from the gel to the PVDF membrane or with the binding of the anti-E-tag antibody to the scFv. This possible interference from storage protein(s) was further investigated by performing Western blot analysis on a bacterially-produced scFv, with or without mixing it with heat-denatured extracts from non-transgenic endosperm tissue. This scFv was slightly larger in size compared to the rice-scFv. This is because the transcribed region retained some sequences from the *E. coli* expression vector resulting in a size difference of 44 amino acids. Results presented in Fig. 4d, e, and f show that while the endosperm protein(s) blocked the detection of rice leaf scFv, this was not the case with the slightly larger, bacterially-produced scFv. Taken together, the results suggest that one or more proteins present in the rice endosperm, that migrate with the rice-scFv on the gel, block its detection on the Western blot.

To confirm that our inability to detect the scFv in the presence of endosperm extract is particularly a limitation of the Western blotting technique, we conducted ELISA on extracts from embryo and leaf tissues with or without mixing with endosperm extract from non-transgenic rice seeds. The results from this analysis are presented in Fig. 5a. Unlike the case with Western blot analysis, endosperm extract did not interfere with the detection of scFv in either the embryo sample or the leaf sample in this ELISA. To further determine how much scFv was produced in various parts of the seed, we performed ELISA on extracts from whole seeds, isolated embryos, and endosperm tissues from line K99-scFv-SK-22A (Fig. 5b). ELISA detected 70 ng scFv/seed and 48 ng scFv/embryo in this line. However, no antibody was detected in the endosperm tissue with ELISA. Thus, with the use of this technique, we were able to confirm the presence of anti-K99 scFv in the embryo portion of rice seed.

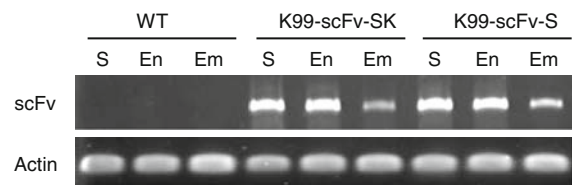
scFv transcripts in rice seeds

The total lack of antibodies in the endosperm tissue was somewhat surprising despite the fact that the published studies show a low level activity for the CaMV 35S promoter in this tissue. To ascertain

whether the absence of scFv in the endosperm was due to weak activity of the promoter, we conducted RT-PCR analysis on RNA isolated from whole seeds, isolated endosperms, and embryos at 14 dpa. The results for non-transgenic, K99-scFv-SK (line 37A), and K99-scFv-S (line 2B) plants are shown in Fig. 6. Surprisingly, this analysis showed that endosperm tissue has higher level of transcription activity compared to the embryos. Thus, it appears that the absence of recombinant antibody in the endosperm is



**Fig. 5** ELISA-based detection and estimation of scFv in various rice tissues (mature seed: S, endosperm: En, embryo: Em, leaf: L). **a** Analysis to ascertain if endosperm extract interferes with ELISA-based detection of scFv in embryo and leaf tissues. Total soluble protein extracts from embryos isolated from mature seeds and leaf tissue from transgenic rice plants were analyzed after mixing with either an equal portion of extraction buffer (Em + B, L + B) or non-transgenic, mature seed-endosperm extract (Em + En and L + En). **b** Quantitation of scFv in seed tissues using ELISA. Levels of scFv in various seed tissues were estimated by comparing their ELISA readings with that of a reading from a known quantity of scFv in the protein extract from rice leaf tissue. Extracts from 20 seeds, isolated endosperm portions or isolated embryos each were used for an individual assay



**Fig. 6** RT-PCR analysis of scFv gene expression in various seed tissues from transgenic rice plants. Total RNA isolated from seed tissues (14 dpa) from wild-type (WT) and two transgenic lines, K99-scFv-SK-37A and K99-scFv-S-2B, were analyzed. Transcripts amplified from rice actin gene served as RNA quantity control. RNA from a pooled sample of five whole seeds (S), endosperm tissues from five seeds (En), and 10 isolated embryos (Em) was used for this analysis

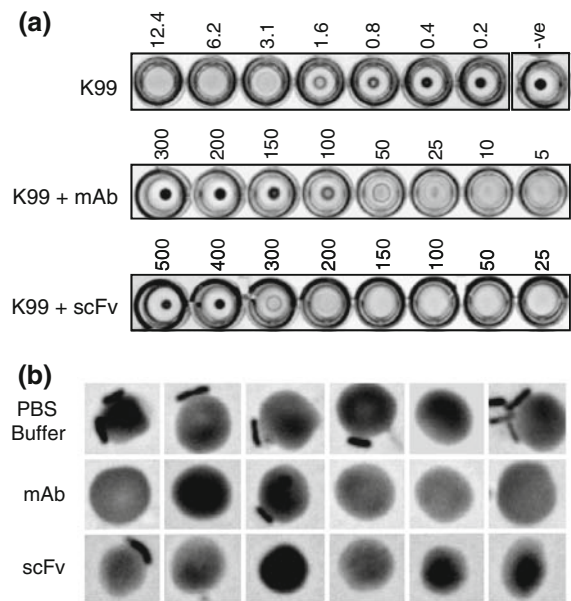


either due to poor translation or protein instability in this tissue.

#### Purified recombinant rice-scFv inhibits k99-induced haemagglutination

It was clear from various Western blot analyses that the levels of the scFv produced in the leaves were substantially higher compared to those in the embryos. Therefore, various analyses for functionality of the rice-produced antibody were conducted with the leaf-derived scFv. Purification with Protein-L column from 2 g of rice leaves yielded 50 µg of purified scFv. Purified K99 protein or ETEC K99<sup>+</sup> can adhere to HRBC and cause haemagglutination. Indeed, recombinant anti-K99 scFv produced in *E. coli* has been shown to inhibit this haemagglutination induced by purified K99 antigen (Bhaskaran et al. 2005). Thus, this system provided a simple yet reliable means of examining the functionality of rice-produced scFv by testing its ability to inhibit K99-induced agglutination of HRBC. To this end, we first optimized the haemagglutination with serially diluted K99 antigen and estimated that a minimum of 3.1 ng K99 is required to render complete haemagglutination (Fig. 7a). Inhibition assays were performed with various concentrations of mAb or scFv and 3.1 ng of K99 protein. Monoclonal antibody and the rice-produced scFv at 25 ng and 200 ng concentrations, respectively, completely inhibited haemagglutination induced by purified K99 protein (Fig. 7a).

We further examined the efficacy of the rice-produced scFv in inhibiting the binding of ETEC to HRBC. The binding assay was carried out with  $5 \times 10^5$  ETEC and  $1.25 \times 10^5$  HRBC in the presence of 0.8 µM mAb or 2 µM scFv. Representative images showing binding of *E. coli* to HRBC as well as the inhibition caused by mAb and scFv are provided in Fig. 7b. The binding efficiency was calculated from this experiment by counting the number of HRBCs that had one or more ETEC attached. The binding efficiencies were 30, 1 and 2% in control, mAb and scFv treatments, respectively. The results from these experiments suggest that the rice-produced scFv is functional and has the ability to interfere with the binding of K99 antigen or K99 expressing *E. coli* cells to the receptors on HRBC.



**Fig. 7** **a** Haemagglutination inhibition assay to ascertain functional properties of rice-produced scFv. Haemagglutination titre was performed with twofold serially diluted K99 protein expressed in *E. coli* (top panel; the number above each well represents nanogram quantity of K99). -ve: PBS buffer. Haemagglutination (induced by 3.1 ng of K99 antigen) inhibition assay was conducted with various concentrations of anti-K99 mAb (middle panel; the number above each well represents ng quantity of mAb tested) or rice-produced scFv (bottom panel; the number above each well represents nanogram quantity of scFv tested). **b** Inhibition of ETEC attachment to HRBC by antibodies. Attachment of ETEC cells to HRBC was observed microscopically, in the presence of PBS buffer as a control (top panel), 0.8 µM anti-K99 mAb (middle panel), or 2 µM rice-produced anti-K99 scFv (bottom panel). A number of HRBCs were photographed under a light microscope at 100× magnification and representative images are depicted in the figure

#### Inhibition of binding of ETEC to calf enterocytes by recombinant scFv

The ability of rice-produced scFv to inhibit the binding of ETEC to calf enterocytes was tested in vitro. The binding assay was performed in PBS buffer with  $8 \times 10^4$  enterocytes and  $5 \times 10^4$  cells of ETEC K99<sup>+</sup> (strain B41). Inhibition experiments were carried out with 0.8 µM mAb or 2 µM rice-produced recombinant scFv. The efficiency of binding inhibition was measured by counting the number of enterocytes with at least one *E. coli* cell adhered to it. Results of three independent experiments showed the average binding efficiency of ETEC cells to

enterocytes to be  $66 \pm 1.2\%$  (Fig. 8a). However, in the presence of mAb or rice-scFv this average binding efficiency decreased to  $9.3 \pm 0.7\%$  and  $11.3 \pm 2.4\%$ , respectively. Representative images of enterocytes from the buffer control and the two antibody (mAb and scFv) treatments from the inhibition studies are presented in Fig. 8b. The results from these in vitro studies demonstrate clearly that the recombinant scFv produced in rice has the ability to block the binding of ETEC to enterocytes.

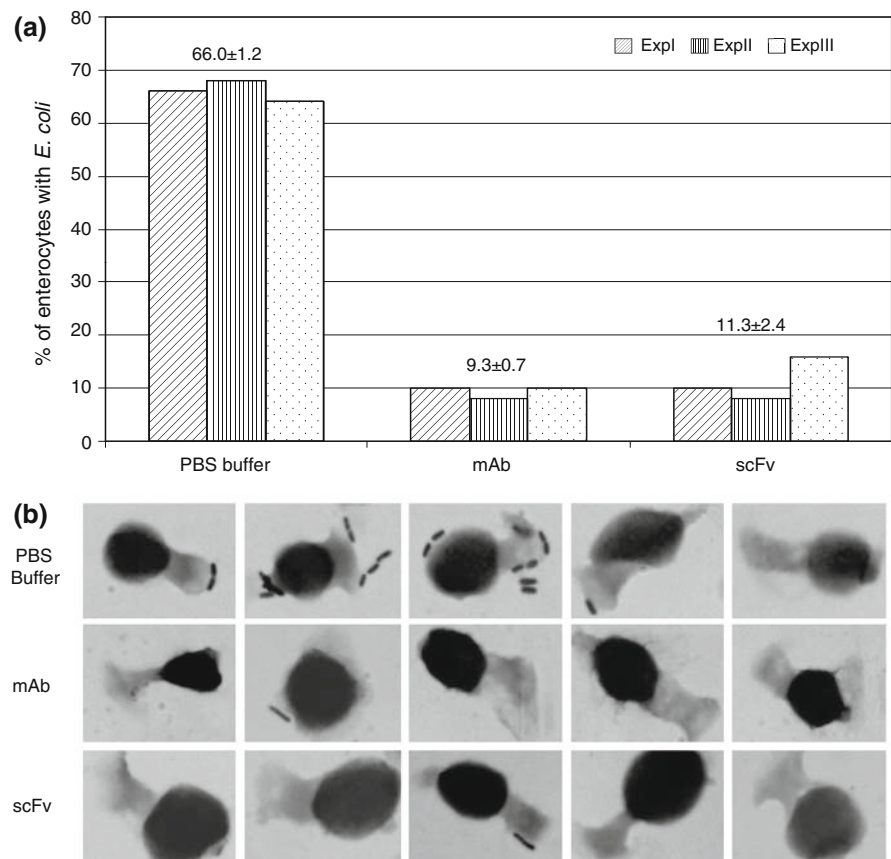
## Discussion

Several studies have reported expression of immunogens in transgenic plants that can be used to address the diseases caused by ETEC. The major subunit of the K88 antigen, FaeG from ETEC that causes pig-scours has been expressed in tobacco (Huang et al. 2003; Joensuu et al. 2004), barley (Joensuu et al. 2006a) and alfalfa (Joensuu et al.

2006b). Recently, soybean was used as a system to express the major subunit of the K99 antigen, FanC (Garg et al. 2007; Piller et al. 2005). The expressed product had the ability to induce anti-K99 antibody in mouse. In this report, we explore an alternative strategy that involves production of anti-K99 scFvs in transgenic rice plants that retain the inhibitory properties of full-length antibodies for use as a passive immunoprophylaxis.

Specifically, the aim of this study was to examine if scFv antibodies against the ETEC surface antigen, K99, can be produced in plants and whether the plant-produced scFv possess the functional characteristics to interfere with the binding of ETEC to the intestinal epithelial cells. As discussed earlier, due to the advantages offered by plants for the production of recombinant proteins, this system has been used to express several therapeutic compounds [Reviewed by (Daniell et al. 2001 and Floss et al. 2007)]. Currently, a number of plant-based therapeutic compounds are being considered for commercialization (Sparrow

**Fig. 8** In vitro assay to evaluate the inhibition of ETEC attachment to calf enterocytes by antibodies. **a** Bar graphs depict results from three independent experiments showing the percentage of enterocytes that had one or more ETEC attached. The data for each bar represent a sample of 100 enterocytes that were observed. The numbers above each set of bars represent average of three independent experiments  $\pm$  SE. **b** Representative images showing inhibition of attachment of the ETEC to enterocytes by mAb and recombinant scFv from rice. Attachment of ETEC cells to enterocytes was observed microscopically, in the presence of PBS buffer as a control (top row), 0.8  $\mu$ M anti-K99 mAb (middle row), or 2  $\mu$ M rice-produced anti-K99 scFv (bottom row)



et al. 2007). In 2006, USDA approved the first plant-produced vaccine that protects chickens from disease caused by Newcastle virus (Vermij 2006). During the early stages of our investigation, expression of the scFv gene constructs was tested in tobacco callus cultures and the reactivity of the recombinant antibody was confirmed by competitive ELISA assay (Data not shown). Since tobacco produces many harmful secondary metabolites, it is not an ideal system to produce pharmaceutical proteins. Therefore, we selected rice as a platform for the production of anti-K99 scFv. In addition to being edible, cereals have other advantages such as the absence of harmful compounds and long-term storability with little or no loss of the recombinant protein activity (Christou et al. 2004). These advantages have motivated others to express antibody genes in transgenic rice such as anti-carcinoembryonic antigen scFv (Stoger et al. 2000; Torres et al. 1999), anti-gibberellins GA<sub>24/19</sub> scFv (Tanaka et al. 2004), anti-Herpes simplex virus 2 IgG (Zeitlin et al. 1998), and chimeric secretory immunoglobulin, sIgA/G (Nicholson et al. 2005). Our report adds one more candidate to the list of antibodies expressed in rice.

Optimal level of expression of recombinant proteins in plant cells is often obtained by directing the protein products into various cellular compartments. In the current study, we tested two different gene constructs each containing a DNA sequence coding for a secretion signal peptide from the tobacco PR1 protein. In addition to the secretion signal peptide, the first construct (K99-scFv-SK) had a DNA sequence encoding the KDEL signal at the 3' end of the gene in order to retain the scFv in the ER (Munro and Pelham 1987; Nilsson and Warren 1994). The second construct, K99-scFv-S, lacking the KDEL coding sequence would allow secretion of the recombinant antibody. Our results show that high levels of scFv were detected only in the K99-scFv-SK transgenic plants. The scFv protein was barely detectable in the K99-scFv-S transgenic plants. However, the results from the RT-PCR analyses revealed the presence of abundant scFv message in the leaves of K99-scFv-S transgenic plants. Thus, the poor antibody levels in these transformants are not due to poor transcription or unstable nature of the message. Results showing the absence of any correlation between the mRNA level and recombinant protein accumulation due to the presence or absence of a C-terminal KDEL

sequence have been noted in some earlier studies (Schouten et al. 1996; Wandelt et al. 1992). Our results are also consistent with several other studies that compared expression levels of ER targeted or secreted recombinant proteins in various plant systems including rice (Stoger et al. 2000), wheat (Stoger et al. 2000), tobacco (Fiedler et al. 1997; Schouten et al. 1996; Wandelt et al. 1992) and alfalfa (Wandelt et al. 1992). Plant ER contains enzymes and chaperones necessary for proper folding of the recombinant proteins (Boston et al. 1996; Denecke et al. 1995; Melnick et al. 1992). In addition, the recombinant proteins can be protected from proteolytic enzymes inside the ER. Therefore, proteins targeted to the ER are expected to be highly stable (Fiedler et al. 1997). These reasons possibly account for substantially higher levels of scFv when targeted to ER in rice.

Although scFv was easily detected in the leaf tissue, our preliminary screen for the detection of scFv in rice seeds using Western blot analysis was unsuccessful. Stoger et al. (2000) reported similar results with the expression of scFv against carcinoembryonic antigen in transgenic rice plants using CaMV 35S promoter. They found that the transgenic rice plants produced scFv in the leaves, but not in the seeds. Using *gusA* gene as a reporter, Terada and Shimamoto (1990) observed that the CaMV 35S promoter was active in rice seeds. However, its activity in the seeds was 12.5-fold lower than the levels observed in the leaves. Taken together, these studies suggested that the CaMV 35S promoter may have very low-level activity in rice seeds. However, the results from the RT-PCR analysis in our study showed that the CaMV 35S promoter is fairly active in the rice seeds. As described in the Results section, this observation prompted us to examine various other possibilities. The results from these additional studies revealed that the scFv was indeed produced in the developing rice seeds and was present in the embryo portion of a mature seed that could be detected by ELISA. However, the seed produced antibodies were not detectable on a Western blot when the protein extract from a 'whole seed' was used for analysis. It is apparent that the endosperm may contain certain factors which inhibit the detection of K99-scFv when using this technique. It is possible that the interfering agents are one or more of the storage proteins which run together with the

rice-scFv on the gel, that either impede the transfer of scFv to the PVDF membrane or block the accessibility of E-tag to the anti-E-tag antibody.

The results obtained in this study also highlight the inconsistencies in the accumulation of a recombinant antibody in three different tissues, i.e., leaf, embryo and endosperm. Although the scFv gene was transcribed in all three types of tissues in rice, the protein was detected only in the leaf and embryo. There may be one or more reasons for the absence of the antibody in the endosperm tissue, including poor translation and targeting to an organelle where the recombinant protein is unstable or is degraded.

The use of CaMV 35S promoter for the production of a recombinant protein in this investigation provided some valuable information regarding the important differences between the embryo and endosperm compartments of the seed. We show here that, in fact, the rate of CaMV 35S promoter-driven transcription for the scFv gene was higher in the endosperm compared to that in the embryo, however, the translational product was not detected in this tissue. Given the nature and organization of the scFv expression cassette used in this study, the rice embryo appears to be more akin to the leaf in terms of its translational and post-translational machinery than the endosperm tissue. There are many possible ways to improve the production and accumulation of recombinant proteins in the endosperm tissue. These include the use of a strong, endosperm-specific promoter (Burkhardt et al. 1997), use of 3' UTR sequences from prolamin or glutelin seed storage proteins (Choi et al. 2000), and codon optimization (Horvath et al. 2000).

Thus, although accumulation of the anti-K99 scFv was not observed in the rice endosperm in the current investigation, it may yet be possible to utilize one or more of the above-mentioned strategies to produce this antibody in the endosperm tissue, the major portion of a monocot seed. If successful, these seeds will offer a cost-effective means to provide large quantities of the antibodies for the protection of newborn calves from the often fatal, colibacillosis.

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