



## Rice cell culture as an alternative production system for functional diagnostic and therapeutic antibodies

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### Abstract

We investigated the suitability of transformed rice cell lines as a system for the production of therapeutic recombinant antibodies. Expression constructs encoding a single-chain Fv fragment (scFvT84.66, specific for CEA, the carcinoembryonic antigen present on many human tumours) were introduced into rice tissue by particle bombardment. We compared antibody production levels when antibodies were either secreted to the apoplast or retained in the endoplasmic reticulum (ER) using a KDEL retention signal. Production levels were up to 14 times higher when antibodies were retained in the ER. Additionally, we compared constructs encoding different leader peptides (plant codon optimised murine immunoglobulin heavy and light chain leader peptides from mAb24) and carrying alternative 5' untranslated regions (the petunia chalcone synthase gene 5' UTR and the tobacco mosaic virus omega sequence). We observed no significant differences in antibody production levels among cell lines transformed with these constructs. The highest level of antibody production we measured was  $3.8 \mu\text{g g}^{-1}$  callus (fresh weight). Immunological analysis of transgenic rice callus confirmed the presence of functional scFvT84.66. We discuss the potential merits of cell culture for the production of recombinant antibodies and other valuable macromolecules.

### Introduction

Antibodies are used as diagnostic and therapeutic reagents for the diagnosis and treatment of human diseases (King, 1996). Their use has increased dramatically with the advent of recombinant antibody (rAb) technology, allowing the production of macromolecules with improved or novel properties. There are several different types of rAb in current use (Plückthun, 1991; Winter & Milstein, 1991). Single chain Fv fragments (scFv) are one of the smallest rAbs (27 kDa). They comprise the variable domains of the heavy and light immunoglobulin chains ( $V_H$  and  $V_L$ ) joined by a flexible peptide linker. Such antibodies have been used

in clinical trials to direct drugs to specific targets or to manipulate the immune system so that it attacks and kills established cancers (Pietersz & McKenzie, 1992). ScFvs are less immunogenic than full sized antibodies because they lack the constant domains and are therefore not glycosylated. Furthermore, they show better tissue penetration, rapid biodistribution, and have minimal assembly and folding requirements, and are useful as immunoreagents (Chester & Hawkins, 1995).

Traditionally, rAbs have been produced in animal cells *in vitro*, transgenic animals or microbial expression systems. However, rAbs produced in animal systems have a number of limitations, including immunogenicity, high production costs and loss of activity during purification. In microbial expression

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systems, antibodies can fold and assemble incorrectly (Spooner et al., 1994; Pen, 1996) and bacterial fermentation often results in the production of inclusion bodies that must be solubilised with harsh chemical treatments (Goddijn & Pen, 1995). Endotoxins produced during bacterial fermentation also add to the cost of antibody production (Fiedler et al., 1997). Some of these problems have been addressed using plant-based expression systems. These show increased cost-efficiency and ease of scale-up (Kusnadi et al., 1997) and could improve biological safety (Logt et al., 1998). ScFvs have been expressed successfully in model plants such as tobacco and *Arabidopsis thaliana* (Conrad & Fiedler, 1994, 1998). This has permitted immunomodulation of metabolic pathways (Owen et al., 1992; Firek et al., 1993; Artsaenko et al., 1995) and increased pathogen resistance in plants (Tavladoraki et al., 1993; Fecker et al., 1996; Zimmermann et al., 1998). In humans, passive therapeutic applications have become possible (Ma et al., 1995). In transgenic plants, scFvs have been shown to accumulate in leaves (Fiedler et al., 1997; Bruyns et al., 1996; Schouten et al., 1996; Firek et al., 1993; Tavladoraki et al., 1993; Owen et al., 1992), roots (Schouten et al., 1997), seeds (Phillips et al., 1997; Fieldler & Conrad, 1995) and tubers (Artsaenko et al., 1998). However, model plant systems such as tobacco produce noxious chemicals (e.g. alkaloids). It is, therefore, important to transfer rAb technology from model systems to species that are safer for rAb production and clinical applications.

rAbs have been expressed in tobacco cell suspension cultures derived from transformed leaf discs (Magnuson et al., 1996; Firek et al., 1993) and tobacco hairy root cells (Wongsamuth & Doran, 1997). Since plants do not have to be regenerated in such *in vitro* systems, and because the controlled environment offers improved safety, cell cultures provide a number of potential advantages over transgenic plants for rAb production. Moreover, the system is amenable to scale-up in fermenters for industrial production. Downstream processing is likely to be less expensive and not as labour-intensive if the antibody can be secreted into the culture medium (Magnuson et al., 1996; Firek et al., 1993).

In this study, we investigated the suitability of rice cell culture for efficient rAb production. We tested a number of expression constructs encoding scFvT84.66 derived from the monoclonal antibody T84.66, which shows high specificity and affinity for CEA, a common tumour-associated antigen expressed in most adeno-

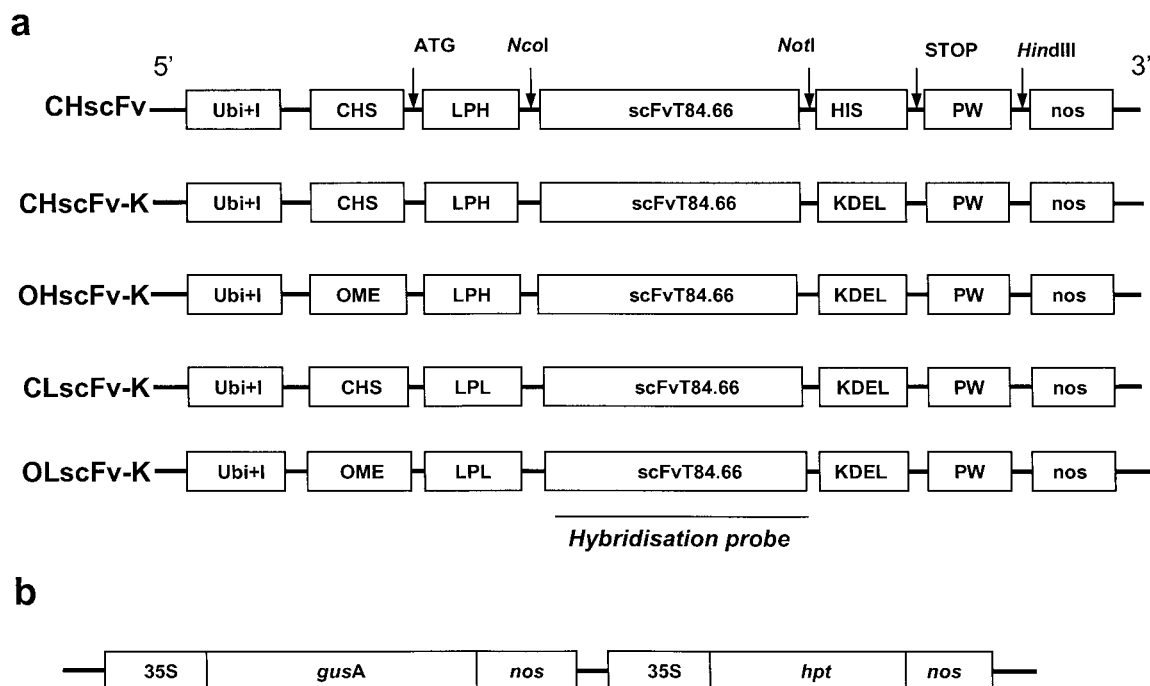
carcinomas of the gastrointestinal tract, breast and lung (Tsang et al., 1995). Although secretion would be preferable in cell cultures for ease of harvesting the product, previous studies have suggested that higher yields are obtained through ER retention (Fiedler et al., 1997). Leader peptides are required for antibody secretion and a number of different leader peptides have been used in previous studies (Zimmerman et al., 1998; Ma et al., 1995; Voss et al., 1995; Hein et al., 1991; During et al., 1990). Similarly, a number of 5' UTR sequences have been shown to act as translational enhancers (Gallie & Walbot, 1992). Initially we compared production levels of antibodies secreted to the apoplast to those retained in the endoplasmic reticulum (ER) using a C-terminal KDEL signal. We then compared two leader peptides (the murine immunoglobulin heavy and light chain leader peptides from mAb24) and two 5' untranslated regions (the chalcone synthase gene 5' UTR and the tobacco mosaic virus omega sequence) for their effects on antibody production.

We demonstrate that a functional therapeutic scFv antibody can be stably expressed at high levels in transformed rice cell cultures. The advantages of this system provide a viable alternative to transgenic plants for the production of recombinant antibodies for diagnostic and therapeutic applications. To our knowledge, this is the first report describing the production of a recombinant therapeutic molecule in a monocot-derived callus cell line.

## Materials and methods

### Expression constructs

The expression constructs used in this study are illustrated in Figure 1. All constructs contained the scFvT84.66 coding region (Wu et al., 1996) driven by the maize ubiquitin-1 promoter and first intron. In all constructs, the tobacco mosaic virus (TMV) pseudoknot region (PW) and *nos* terminator were located downstream of the coding region. Constructs CHscFv and CHscFv-K carried the *CHS* 5' UTR and the murine immunoglobulin heavy chain leader peptide (LPH) between the promoter and the scFv coding sequence. The LPH was derived from the TMV virion-specific mAb24 (Voss et al., 1995). It was codon-optimised for plants according to Angenon et al. (1990) and fused in-frame with the scFv coding region. At the 3' end of the coding region, CHscFv carried a sequence encoding a His<sub>6</sub> tag to allow protein



**Figure 1.** Expression constructs. (a) Schematic representation of the scFvT84.66 constructs used for rice transformation. (b) Plasmid containing the selectable marker *hpt*. Ubi+I = maize ubiquitin-1 promoter and intron; *CHS* = chalcone synthase gene 5' UTR from *Petunia hybrida*; OME = omega sequence from tobacco mosaic virus (TMV). LPH and LPL = heavy- and light-chain leader peptides from murine IgG; HIS = histidine tag; KDEL = ER retention signal; PW = pseudoknot region of TMV; nos = nopaline synthase terminator; 35S = cauliflower mosaic virus 35S promoter; *gusA* =  $\beta$ -glucuronidase gene.

purification by immobilised metal ion affinity chromatography (IMAC), while CHscFv-K carried the KDEL endoplasmic reticulum (ER) retention signal. The remaining three constructs carried the 3' KDEL motif, but differed with respect to the 5' UTR and leader peptide sequences. OHscFv-K carried the TMV omega sequence and the heavy chain leader peptide; CLscFv-K carried the *CHS* 5' UTR and the light chain leader peptide (LPL), the latter also derived from mAb24 (Voss et al., 1995) and codon-optimised for plants (Angenon et al., 1990). Finally, OLscFv-K carried the TMV omega sequence and the LPL. Rice callus was co-transformed with a plasmid containing the *hpt* (hygromycin phosphotransferase) gene driven by the CaMV 35S promoter (Figure 1).

#### Plant material and transformation

Scutellum-derived callus from mature rice seed (*Oryza sativa* L. cv. Bengal) was transformed by particle bombardment as previously described (Klein et al., 1987; Sudhakar et al., 1998). Each scFvT84.66 plasmid was mixed with the plasmid carrying the selectable marker at a molar ratio of 3:1. Preparation of DNA-coated

gold particles for bombardment was described previously (Christou & Ford, 1991). Six weeks after bombardment, hygromycin resistant (*hyg*<sup>r</sup>) callus was recovered. Callus lines were subcultured twice (at 20-day intervals) on selective medium.

#### Analysis of scFvT84.66 production by ELISA

Three protein samples were extracted from each callus line for ELISA analysis. The first sample was taken six weeks post-bombardment and subsequent samples at three-week intervals. Extraction of total soluble protein from 100 mg tissue samples was carried out as described by Fischer et al. (1998). Functional scFvT84.66 was quantified by competition ELISA with the full-size murine T84.66 monoclonal antibody. Microtitre plates (Dynex Technologies) were coated with 500 ng ml<sup>-1</sup> recombinant CEA/NA3 antigen (You et al., 1998) in bicarbonate buffer and blocked with 150  $\mu$ l 1% w/v bovine serum albumin in saline (0.85% NaCl, pH 7.2). Plates were washed three times between each step with PBST. Transgenic callus protein extracts were diluted with extracts from wild-type rice tissue. Serial dilutions were prepared

and 2.5 ng full-sized murine T84.66 antibody was added to 100  $\mu$ l aliquots of each dilution in siliconised plates. These were transferred to the CEA/NA3-coated ELISA plates, and the incubation was carried out for 1 h at 37°C. Alkaline phosphatase (AP)-conjugated IgG Fc-specific goat anti-mouse (100  $\mu$ l of a 1:5000 dilution; Jackson Immunoresearch) was added to each well and incubated for 1 h at 37°C. Detection was carried out using 100  $\mu$ l AP substrate buffer (1 mg ml<sup>-1</sup> *p*-nitrophenylphosphate in 0.1 M diethanolamine, 1 mM MgCl<sub>2</sub>, pH 9.8; Sigma). The absorption at 405 nm was determined using a Spectra Max 340 spectrophotometer (molecular devices). The dilution required for 50% inhibition of the colourimetric reaction (compared to the OD<sub>405</sub> obtained with the wild type extract) was used to determine scFvT84.66 activity. We assumed equimolar concentrations at 50% inhibition and took the relative molecular masses of scFvT84.66 and full size mAb T84.66 into account for the calculation. The mean value is the average production level using each construct, obtained from three assays performed at different subculture intervals.

#### *Southern and northern blot analyses*

DNA was isolated from callus according to the method of Dellaporta et al. (1984). Aliquots of DNA (10  $\mu$ g) were digested with *Hind*III (which cuts once in the expression vectors; Figure 1) and fractionated by 0.8% agarose gel electrophoresis. The DNA was blotted onto nylon membranes (Hybond-N<sup>+</sup>, Amersham) and hybridised according to standard methods (Sambrook et al., 1989). A [<sup>32</sup>P]-labelled hybridisation probe, comprising the coding region of scFv T84.66 cDNA, was prepared using the random primer labelling kit (Gibco-BRL). Total RNA was isolated from callus lines using the QIAgen RNA isolation kit. Aliquots (10  $\mu$ g) were fractionated on denaturing 1.2% agarose formaldehyde gels (Sambrook et al., 1989) and blotted onto nylon membranes (Hybond-N, Amersham). Even loading of RNA was confirmed by hybridisation with a ribosomal RNA probe. Hybridisation was carried out as for Southern blots.

## **Results and discussion**

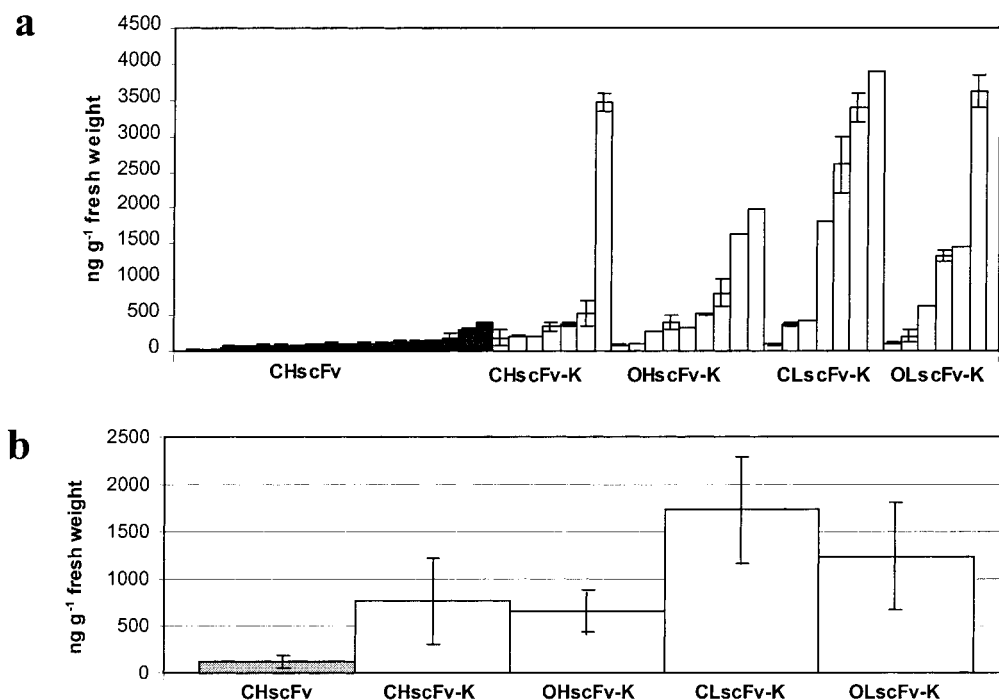
#### *Comparison of KDEL<sup>+</sup> and KDEL<sup>-</sup> constructs*

To compare the effects of secretion and ER retention on antibody production levels, rice callus was transformed with either CHscFv-K (KDEL<sup>+</sup>) or CHscFv

(KDEL<sup>-</sup>). Six weeks after bombardment, we recovered seven hygromycin resistant (hyg<sup>r</sup>) callus lines expressing CHscFv-K and 18 hyg<sup>r</sup> lines expressing CHscFv. ScFv production levels were quantified by competitive ELISA, as described in Materials and methods. Three assays were performed for each line, with samples taken at three-week intervals. We observed a significant difference in production levels between lines transformed with KDEL<sup>+</sup> and KDEL<sup>-</sup> constructs (Figure 2a). The average production level for the KDEL<sup>+</sup> cultures was 680 ng g<sup>-1</sup> fresh weight, whereas that for the KDEL<sup>-</sup> cultures was 150 ng g<sup>-1</sup> fresh weight. Our standard error calculation of the mean value for the different lines confirmed that the difference between KDEL<sup>+</sup> and KDEL<sup>-</sup> was statistically significant (Figure 2b). The highest production level we measured was 3.8  $\mu$ g g<sup>-1</sup> fresh weight in a line transformed with CHscFv-K (Figure 2a).

CHscFv and CHscFv-K integration patterns were compared by Southern blot hybridisation (Figure 3). The integration patterns were unique among the different lines, confirming their independent origin. The results indicated no correlation between transgene copy number and the level of antibody production, that is, similar antibody levels were found in plants containing different transgene copy numbers. These results were consistent with previous reports from our laboratory (Kohli et al., 1999; Bano & Christou, 1999).

We found that production levels were up to 14 times higher when the antibody was retained in the ER rather than secreted to the apoplast. Direct comparison of the production levels of secreted and retained antibodies have shown up to a 100-fold increase when a retained anti-cutinase scFv antibody was expressed in transgenic tobacco plants or cell lines (Schouten et al., 1996). Northern blot analysis was carried out on six representative lines using the scFvT84.66 cDNA fragment as a probe (Figure 4). Equal RNA loading was confirmed by hybridisation with a ribosomal RNA probe. This showed comparable steady state mRNA levels with both constructs we used, suggesting that the higher levels of antibody production observed with the KDEL<sup>+</sup> construct reflected enhanced protein synthesis or accumulation. The KDEL sequence facilitates antibody accumulation in the ER. Antibody fragments passing through the ER *en route* to the apoplast are only transiently exposed to the appropriate molecular chaperones, so a proportion of them could be incorrectly folded. Alternatively, the ER could protect the antibodies from proteolytic activity, resulting



**Figure 2.** ScFv T84.66 antibody production levels in stably transformed rice callus lines. (a) Lines transformed with CHscFv, CHscFv-K, OHscFv-K, CLscFv-K and OLscFv-K. Bars represent mean values  $\pm$  standard error for each line. The mean value is the average production level obtained from three assays performed at different subculture intervals. (b) Effect of KDEL retention signal on mean antibody production (CHscFv and CHscFv-K) and comparison of the two leader peptides (murine IgG heavy and light chain leader peptides) and the two 5' UTRs (CHS 5' UTR and the TMV omega sequence) on antibody production (CHscFv-K, OHscFv-K, CLscFv-K and OLscFv-K). Bars represent mean values  $\pm$  standard error. The mean value is the average production level in the lines transformed with each construct.

in a lower rate of protein turnover compared to that in the apoplast (Fiedler et al., 1997).

The relatively low level of antibody secretion from callus lines does not preclude their use in fermentation. Indeed, the purification of nonsecreted scFvs could be achieved with relatively few extra purification steps, compared to leaves or seeds. Moreover, if high protein turnover occurs in the apoplast, improved culture conditions may improve the stability of secreted scFvs. Culture conditions can significantly influence the concentration of a biologically active foreign protein secreted into the apoplast by plant suspension cultures (Magnuson et al., 1996). The recovery of a mammalian protein secreted from tobacco suspension cultures was increased from 10 to 360 ng protein per ml when the protein stabilising agent polyvinylpyrrolone was added. Preliminary results from suspension cultures derived from our transgenic callus producing up to  $3.8 \mu\text{g g}^{-1}\text{FW}$  showed that scFvT84.66 was released into the culture medium, most likely due to cell lysis. Four weeks post-inoculation, we detected active scFv by competitive ELISA in the culture supernatant at levels up to  $1 \mu\text{g ml}^{-1}$ . The antibody had not been

detected in the medium until this time, suggesting it might be released when the cells reached stationary phase. The release of stored scFv from the ER can be exploited for the enhanced production of antibodies in cell suspension cultures.

#### *Comparison of different leader peptides and 5' UTRs*

We used four KDEL<sup>+</sup> expression constructs carrying all four possible combinations of the two different leader peptide sequences (murine immunoglobulin heavy and light chain leader peptides) and the two different 5' UTRs (chalcone synthase gene 5' UTR and TMV omega sequence). These constructs are illustrated in Figure 1. We compared antibody production levels in callus transformed with each construct. Eight weeks after bombardment we recovered 29 hyg<sup>r</sup> lines (seven CHscFv-K, nine OHscFv-K, seven CLscFv-K and six OLscFv-K; Figures 2a and 2b).

ScFv production levels were determined by competitive ELISA, and the results showed no statistically significant differences among the four constructs (Figure 2a). However, we observed a trend

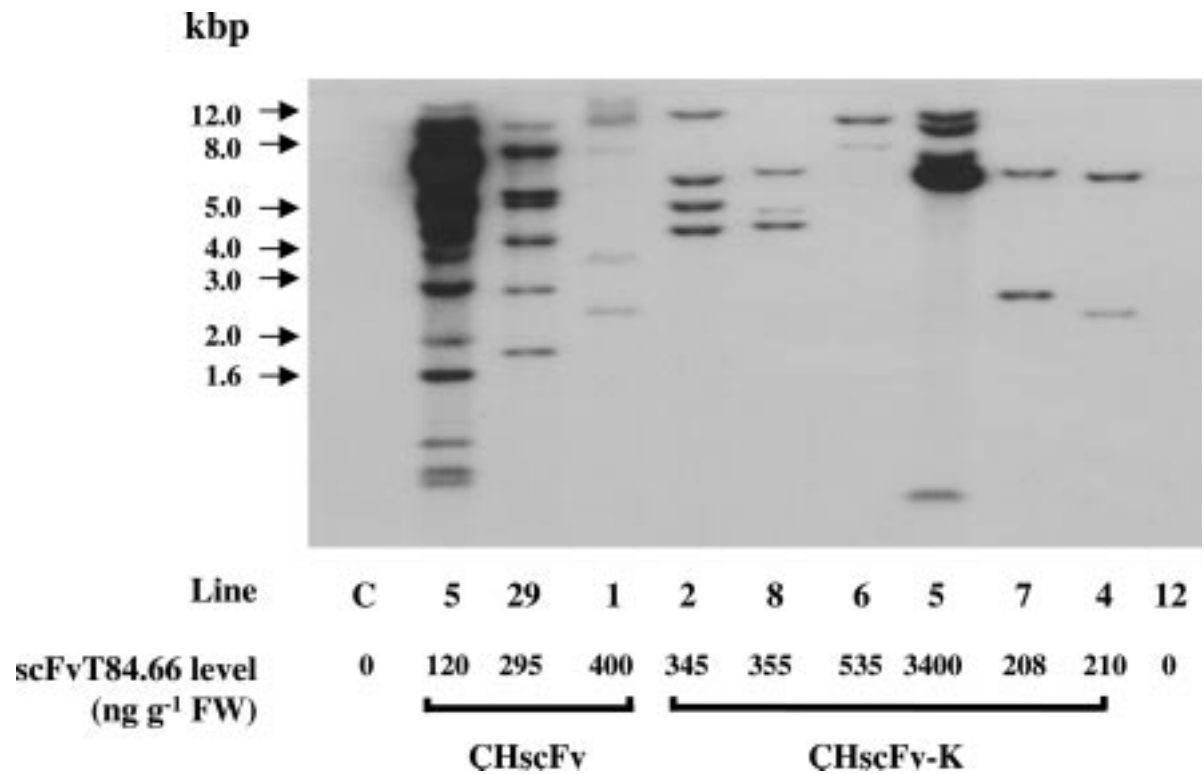


Figure 3. Southern blot analysis of transgenic rice callus lines. Genomic DNA was digested with *Hind*III, which cuts only once in the plasmid. The scFvT84.66 coding region was used as the probe. Transgenic lines 5, 29, and 1 were transformed with CHscFv. Transgenic lines 2, 8, 6, 5, 4, and 7 were transformed with CHscFv-K. Transgenic line 12 was transformed with the selectable marker only. C = wild type callus.

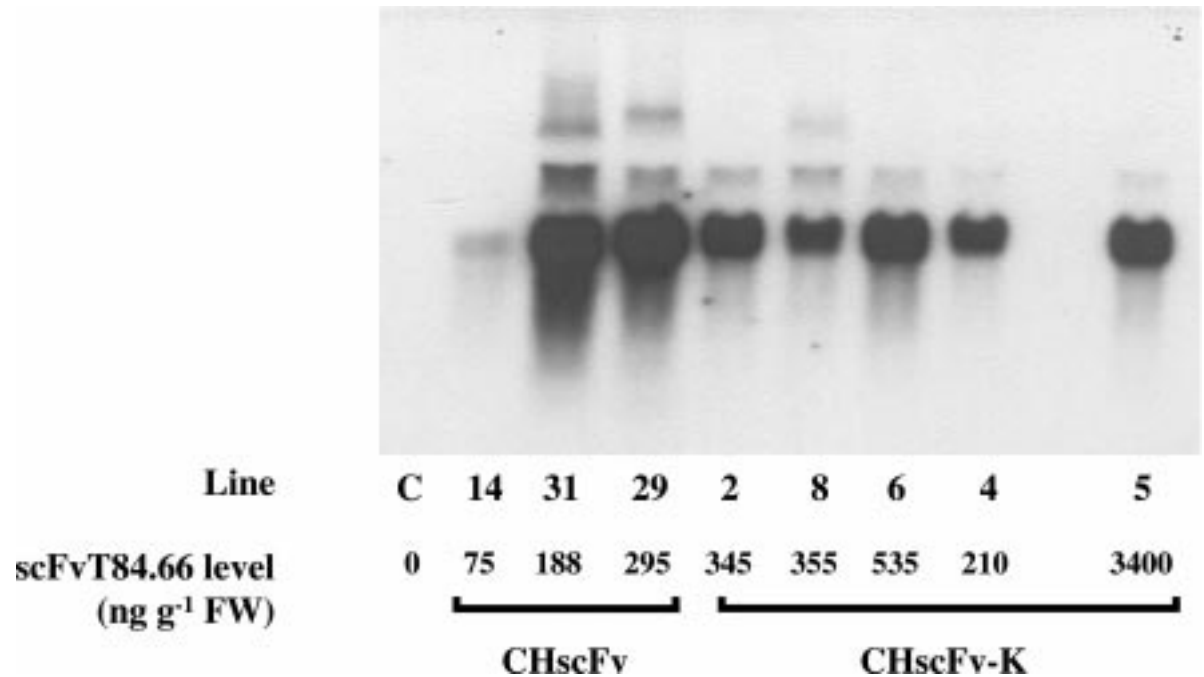


Figure 4. Northern blot analysis of transgenic callus lines. Transgenic lines 14, 31, and 29 were transformed with CHscFv. Transgenic lines 2, 8, 6, 4, and 5 were transformed with CHscFv-K. C = wild type callus.

towards higher production levels using the plant codon optimised LPL (Figure 2b). The average antibody production levels were 650 ng g<sup>-1</sup> (CHscFv-K), 600 ng g<sup>-1</sup> (OHscFv-K), 1800 ng g<sup>-1</sup> (CLscFv-K) and 1200 ng g<sup>-1</sup> (OLscFv-K) FW (Figure 2b). Three lines produced ca. 3.5 µg g<sup>-1</sup> (Figure 2a).

These results suggested that, among the elements investigated in our study, the nature of the leader peptide and translational enhancer sequences were not critical determinants of efficient scFvT84.66 production. The nature of the leader peptide was important for the efficient secretion of some recombinant proteins in plants (Hiatt et al., 1989; Chrispeels, 1991; During et al., 1990; Hein et al., 1991). We found that the heavy and light chain leader peptides were equally efficient in terms of antibody production. The presence of the 68 nt TMV omega sequence upstream of the transgene coding region has been shown to increase translational efficiency 3- to 11-fold in maize and rice protoplasts (Gallie et al., 1989). The 5' UTR of the *Petunia* chalcone synthase gene has improved translational efficiency in tobacco cells (Voss et al., 1995; Zimmerman et al., 1998). Our results suggest that the omega sequence and *CHS* 5' UTR are equivalent in terms of their effect upon antibody production in stably transformed cell lines.

In conclusion, we have shown that a functional therapeutic scFv antibody can be stably expressed at high levels in transformed rice cell cultures. The availability of a homogeneous rice cell suspension line permits antibody production by fermentation, using similar techniques and equipment to those for the fermentation of lower eukaryotes. Plant cell suspensions can be cultivated using conventional fermentation equipment, with minor adjustments, and standard modes like batch, fed-batch, perfusion and continuous fermentation (Schlatmann et al., 1996; Ten Hoopen et al., 1992; Hooker et al., 1990). Significantly, rice is a well characterised food crop that does not produce any of the toxic secondary metabolites found in model cultures, such as tobacco.

The capacity of plant suspension cells to synthesise, process and target large, complex mammalian proteins makes them an attractive system for recombinant protein production. Further benefits of using plants are the reduced technical, ethical and safety issues and costs compared to mammalian cell cultures or transgenic animals. When clinical use of recombinant proteins is intended, their production under defined, controllable and sterile conditions in a fermenter with straightforward purification protocols is advantageous.

Therefore, expression of recombinant antibodies in transgenic rice cell suspensions using shake flask or fermentation cultures will be an important development.

A great advantage of plant cell suspension cultures is that recombinant proteins can be produced under certified conditions (GMP, GLP). Recent improvements in the design of novel promoters (Ni et al., 1995) and gene targeting will lead to significant improvements in recombinant product yields in plant cell lines that are devoid of noxious compounds. However, media optimization, improvement of nutrient supply (Sakamoto et al., 1993; Sato et al., 1996), feeding of precursors or elicitors (Yukimune et al., 1996), improved fermenter design and agitation conditions (Böhme et al., 1997), need to be evaluated to obtain higher productivity during extended use of plant cell suspension cultures in recombinant protein production.

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