J Rubb Res, **5(4)**, 215–225

Expression of a Functional Recombinant Antibody Fragment in the Latex of Transgenic Hevea brasiliensis

H Y YEANG*#, P AROKIARAJ*, HAFSAH JAAFAR*, SITI ARIJA M ARIF*, S RAJAMANIKAM*, J L CHAN*, JAFRI SHARIB*, R LEELAVATHY*, SAMSIDAR HAMZAH* AND C P E VAN DER LOGT**

Hevea *genetic transformation was carried out by* Agrobacterium *mediation using a gene construct for an antibody single chain variable fragment (scFv) against the coat protein of the bacterium* Streptococcus gordonii (Streptococcus sanguis) *Gene expression was controlled by the* 35S CaMV *promoter and* nos *terminator regulatory sequences, together with the tobacco pathogenesis-related protein* prla *signal sequence The gene construct also incorporated the marker* nptII *for transgene selection and the hydrophil-2 detection tag to monitor protein expression Out of over 30 transgenic plants successfully transferred to the soil, 18 were checked for the presence of the* scFv *cDNA by PCR All the transgenic plants were found to be positive for these inserts, whereas the four control plants tested were negative The presence of the scFv protein in latex obtained from transgenic and control plants were analysed by ELISA The scFv protein was detected in the latex from all the 11 transgenic plants tested while none of the four control plants tested positive When the assay was modified so that the recombinant antibody bound to the antigen coated on the ELISA plate, six of the 11 transgenic plants were positive, indicating that these antibody fragments were functional The recombinant proteins synthesised in the latex of the other five plants functioned poorly as antibodies Hence, although all the 11 transgenic plants harboured the* scFv *gene, expression of the protein varied between the individual plants in quantity and functionality Concentration of the scFv protein in the latex increased as the transgenic plants aged*

Key words: *Hevea brasiliensis*, *Streptococcus gordonii*, genetic transformation, antibody, scFv, latex, protein

While one of the best known applications of modern plant biotechnology is in genetic transformation to enhance crop productivity, plant genetic engineering has in fact another promising application to transform plants into living factories for the production of valuable recombinant proteins The synthesis of commercially important proteins, particularly

*Rubber Research Institute of Malaysia, Malaysian Rubber Board, P O Box 10150, 50908 Kuala Lumpur, Malaysia
**Unilever Research and Development Vlaardingen, Olivier van Noortlaan 120, 3133AT Vlaardingen The Netherlands
#Corresponding author (e-mail hyyeang@lgm gov my)

pharmaceuticals, by mirco-organisms (commonly, bacteria and yeasts in bioreactors) involve processes well entrenched in industry. More recently, DNA engineering in animals has enabled the expression of foreign proteins (commonly, therapeutic proteins) in the milk of animals such as sheep, goats and cows[1]. These animals effectively become natural bioreactors that support the sustained yield of target proteins which include human hormones, enzymes, blood coagulating factors and immunological agents. Like transgenic animals, plants can be genetically transformed to express protein-based pharmaceuticals and other valuable proteins[2]. Plants are cheaper to maintain than animals and their multiplication though inbred seeds is relatively simple and efficient. An important characteristic of many plants is their amenability to vegetative propagation for clonal multiplication. Grown in the field, plants require little more than sunlight, water and basic horticultural input to thrive. As protein manufacturing factories, plants are solar-powered and ecologically friendly.

The many advantages of transgenic plants for 'bio-pharming' notwithstanding, their one significant weakness is the difficulty in recovering the recombinant protein. Unlike transgenic animals where there is continual protein production in the milk, harvesting of the recombinant protein involves destruction of the plant or a portion of it. As a result, protein recovery is not usually a continual process. The drawbacks of transgenic animals are in maintenance costs and the difficulty in multiplying them clonally. In these respects, *Hevea brasiliensis* offers the advantages inherent in both plants and animals.

The rubber tree produces voluminous latex which can be extracted non-destructively through the process of tapping. By transforming *Hevea* with genes that control the production of high-value proteins, transgenic rubber plants could serve as efficient, low cost, low maintenance and environment-friendly production lines for the production of the targeted proteins[3]. The transgenic rubber tree then becomes essentially a living 'factory'. The system would enable continual harvesting of the protein exuded in the latex that is free of bacteria and animal viruses. As *Hevea* is amenable to vegetative propagation, large numbers of genetically identical plants can be multiplied from a single selected transformant. Examples of commercially valuable proteins are those employed in pharmacology and personal care products.

Hevea callus tissue has been successfully transformed with the genes for kanamycin resistance, GUS and CAT by co-cultivation with *Agrobacterium*[4,5] and by the use of the particle gun[6]. The *gus* gene is expressed in the leaf of the transgenic plant and, crucially, also in the latex obtained from the transgenic plant regenerated through *in vitro* culture. In fact, GUS expression is especially enhanced in the latex within latex vessels[5]. The expressed GUS protein is found in the aqueous latex serum which can be efficiently recovered by centrifugation.

This paper reports the genetic transformation of the cDNA encoding an antibody single chain variable fragment (*scFv*) into *Hevea*. This recombinant antibody fragment, recognises the coat protein of the bacterium *Streptococcus gordonii*, the predominant organism in early dental plaque. The variable region genes of a monoclonal antibody (designated 4715) developed against this bacterium has been successfully synthesised in microbial systems[7]. The *scFv* fragment was very effective in the delivery of antibacterial enzymes that rapidly killed the targeted microbes. The antibody fragment has also been successfully expressed

in plants[7] However, extraction and recovery of the target protein has remained a problem because of the low yield and the fact that destructive harvesting was involved With the transgenic *Hevea* system, recovery of the recombinant protein from the latex that is extracted by tapping can be expected to be comparatively simple

MATERIALS AND METHODS

The plasmid for genetic transformation contained the gene for a mouse immunoglobulin single chain variable fragment (*scFv*) with specificity for the dental bacterium *Streptococcus gordonii* (formerly *Streptococcus sanguis*) The synthetic antibody consisted of the heavy and light chain domains of the immunoglobulin joined by a linker peptide The *35S CaMV* (cauliflower mosaic virus) promoter and *nos* (nopaline synthase) terminator regulatory sequences and the selectable marker *nptII* (neomycin phosphotransferase II), together with the coding region of the tobacco pathogenesis-related protein prla signal sequence regulated expression of the recombinant antibody fragment A detection tag (hydrophil-2) was attached to facilitate the monitoring of protein expression in the latex The schematic diagram of the gene construct is depicted in *Figure 1* The gene construct was transformed into *Agrobacterium tumefaciens* (GV3850)

Hevea tissue culture and genetic transformation *via Agrobacterium* mediation has been carried out by Arokiaraj *et al*[4] on the clone GI 1 The presence of the inserted genes in putative transgenic plants was analysed by the polymerase chain reaction (PCR)[8] The primers used for amplification of *nptII* and *ScFv 4715* were 5'-gaggctattcggctatgactg-3', 5'-atcgggagcggcgataccgta-3' and 5'-ggatgggatttgttctcttttca-3', 5'-ggcttcaggtaccccta-3', respectively

To sample latex from young *Hevea* plants, the stem of the plant was cut with a razor blade and the exuded latex collected in 20 mM Tris-HCl buffer, pH 8, containing 150 mM NaCl (Tris buffered saline, TBS) and 10 μg mL^{-1} leupeptin After sonication, the latex aqueous phase was recovered after centrifugation at 24 000 g The latex serum so obtained was therefore a mixture of the latex C-serum and the latex B-serum that would have been released from the lutoids during sonication In determining the protein content in the serum, the total solid content of the latex was also determined to correct for variation in the solids (mainly rubber) that would affect the proportion of serum present in the latex

Quantitation of the recombinant proteins present in the latex was by ELISA[9] Three variants of the technique, *Formats 1*, *2* and *3* were employed In *Format 1*, test samples or calibration standard protein (scFv 4715 protein originating from transformed bacteria) were diluted 1/10 with 0 1 M bicarbonate-carbonate buffer, pH 9 8, and pipetted into the wells of the ELISA plates in triplicate After overnight incubation at 37°C, the plates were blocked with 1% bovine serum albumin in TBS for 1 h In the case of the calibration standard, two-fold serial dilutions were carried out until a concentration of 0 016 μg mL^{-1} was reached After incubation at 37°C for 2 h, the plates were washed three times with TBS containing 0 1% Tween 20 (TBS-T) To detect scFv 4715, a monoclonal antibody (1 μg mL^{-1}) that recognised the hydrophil-2 fragment of the scFv protein was added and incubation was carried out at 37°C for 90 min After washing with TBS-T, the monoclonal antibody was allowed to react with the secondary antibody, anti-mouse IgG conjugated to alkaline phosphatase,

Figure 1. The gene construct PRIscFv.4715 used in genetic transformation, comprising nptII (neomycin phosphotransferase II), p-nos (nopaline synthase promoter), 35S (35S cauliflower mosaic virus promoter), pr1a (tobacco pathogenesis-related protein signal sequence), VH (variable heavy chain), linker, VL (variable light chain), hydro-2 (hydrophil-2 detection tag) and NOS-T (nopaline synthase terminator).

for 1 h at 37°C. The plate was then washed with PBS-T followed by TBS. The secondary antibody was detected by the addition of disodium *p*-nitrophenylphosphate that produced a coloured product, the absorbance of which was read at 405 nm on a microplate reader. Absorbance readings exceeding the blank by more than 2 x its standard deviation were considered to be significantly above background. In determining the concentration of recombinant protein in the latex serum, corrections to the readings were made to allow for dilution by the extraction buffer and for the presence of rubber in the latex.

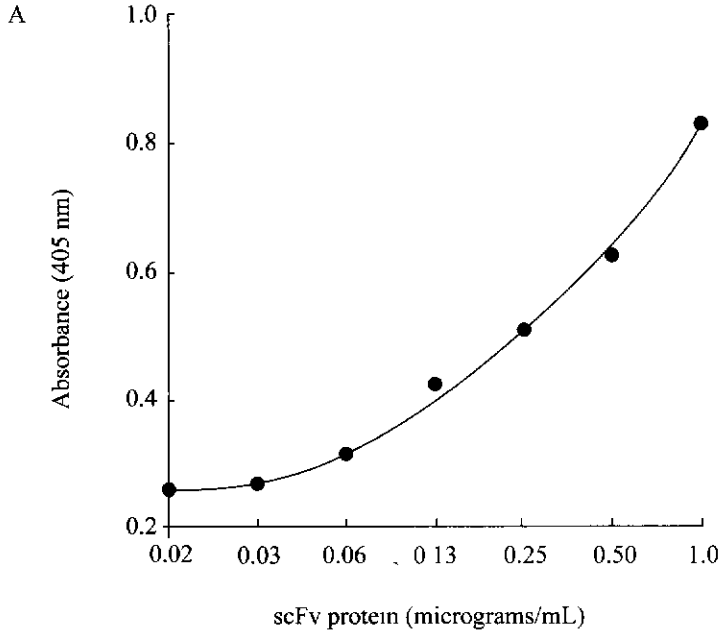
In *Format 2* ELISA, the ELISA plates were coated with a culture of *Streptococcus gordonii* diluted 1/10 with 0.1 M bicarbonate-carbonate buffer, pH 9.8, and incubated overnight at 37°C. The plates were blocked with 1% bovine serum albumin in TBS for 1 h before addition of test or calibration standard samples as in *Format 1*. All subsequent procedures were as for *Format 1*. Calibration curves for ELISA *Formats 1* and *2* are shown in *Figure 2*.

Format 3 was a two-site ELISA in which the monoclonal antibody against the hydrophil tag of the scFv protein served as the capture antibody and a polyclonal antibody against the same protein acted as the detection antibody. The former was diluted in bicarbonate-carbonate buffer and coated on the ELISA plate for overnight incubation before reaction with test or calibration standard samples. The polyclonal antibody against the hydrophil tag was added and binding to the scFv protein was detected using an anti-rabbit IgG secondary antibody linked to alkaline phosphatase.

To carry out immuno-blotting, proteins in latex extracts were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 15% gels according to the method of Laemmli[10] and Western blotted on to nitrocellulose membrane. Immuno-detection of the scFv 4715 recombinant protein on the nitrocellulose membrane was carried out using standard procedures[11]. The monoclonal antibody against the hyrophil-2 tag served as the primary antibody while alkaline phosphatase conjugated to the secondary antibody generated the colour enzyme product for protein visualisation.

RESULTS

Hevea genetic transformation was carried out with a gene construct that embodied the cDNA encoding an antibody single chain variable fragment (*scFv*). Transformation success of calli derived from anthers was 3.9%. From the kanamycin-resistant calli that were recovered, embryogenesis was 546% (2057 embryoids from 377 kanamycin-resistant calli) while plantlet regeneration was 4.2%. In an initial



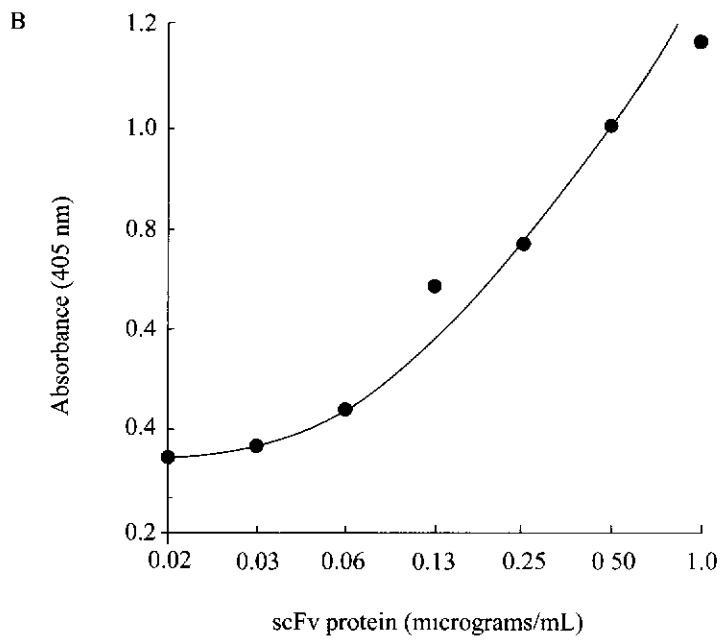


Figure 2. Calibration curves for scFv protein as determined by ELISA Format 1 *(A) and* Format 2 *(B). Results are the means of triplicate readings.*

set of experiments, thirty transgenic plants were successfully transferred to the soil (50% success). Leaves from 11 of these plants were checked for the presence of the *nptII* (kanamycin-resistance) DNA and 18 were analysed for the presence of the *scFv* DNA by the polymerase chain reaction (PCR). All the transgenic plants were positive for these inserts, whereas the four control plants tested were negative (*Figures 3* and *4*).

The presence of the scFv protein in latex obtained from the 11 plants (22 to 23 months after transplanting into soil) and four untransformed control plants of the same age were analysed by ELISA in three formats:

1. One-site ELISA, where the scFv protein was coated on to the ELISA plate and the monoclonal antibody against the hydrophil tag was used as the detection antibody.

2. Two-site ELISA, where the antigen (*Streptococcus gordonii*) was coated on to the ELISA plate, followed by the scFv protein which should bind to the antigen. The monoclonal antibody against hydrophil tag was then used as the detection antibody.

3. Two-site ELISA where the monoclonal antibody was coated on to the ELISA plate to serve as the capture antibody, followed by the scFv protein. The polyclonal antibody against the hydrophil tag was then used as the detection antibody.

Format 3 was carried out as a preliminary test basically to confirm that the positive ELISA signals were specific for the hydrophil tag, and that they did not result from irrelevant cross-reactions (results not presented).

The scFv protein was detected in the latex from all the 11 transgenic plants analysed using the one-site ELISA (*Format 1* above), but the levels of expression were variable, spanning six-fold between the highest and lowest values (*Figure 5A*). None of the four control plants tested positive, all of them showing absorbance readings below that of the lowest scFv calibration standard used to construct the standard curve (equivalent to 0.04 μg mL^{-1}). A positive latex extract was Western blotted following SDS-polyacrylamide gel electrophoresis. Immuno-detection using the monoclonal antibody against the hydrophil tag revealed the presence of protein bands of about 34 kDa to 36 kDa that were absent in the control extracts. A faint band of 37 kDa was also observed. In comparison, the scFv protein positive control migrated as a 37 kDa peptide on the gel (*Figure 6*). Besides the principal protein bands, non-specific binding of the antibody to other irrelevant proteins was noted in both transgenic and control plants.

For a commercial product to be viable, the harvested protein must retain the salient characteristics of the native protein. In the case of a recombinant antibody, it was essential that the protein could recognise and bind to its antigen. Whereas *Format 1* ELISA quantitated the amount of scFv protein present in the latex, *Format 2* was carried out to determine whether the recombinant antibody fragment was functional in being able to bind to its designated antigen. Comparing *Figures 5A* and *5B*, it is seen that *Format 2* readings are higher than corresponding readings from *Format 1*. The scFv protein of *Hevea* origin may have higher immuno-reactivity to the *Streptococcus* antigen than the bacterial scFv used to construct the calibration curve. This would give relatively higher readings in *Format 2* ELISA that involved affinity between the scFv

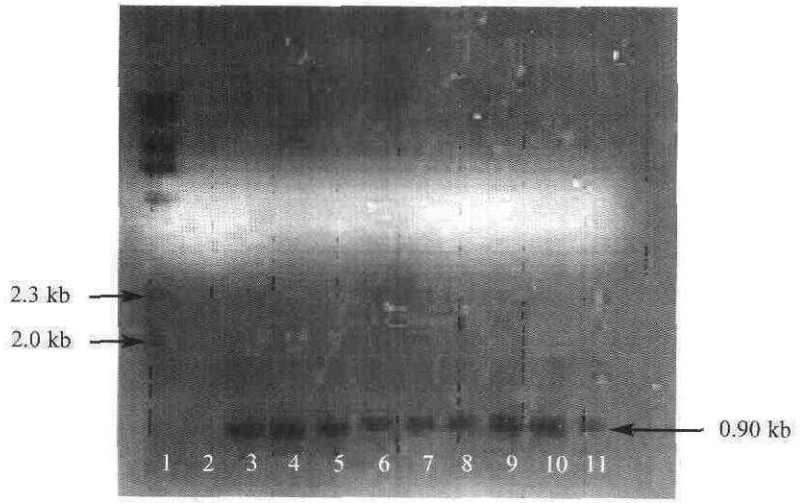


Figure 3. Amplified fragment of PRIscFv.4715 *cDNA (0.9 kb) from leaves.*
Lane 1: Lambda/Hind III marker; Lane 2: Control plant; Lane 3: Positive control (PRIscFv.4715); Lanes 4 to 11: Transgenic plants.

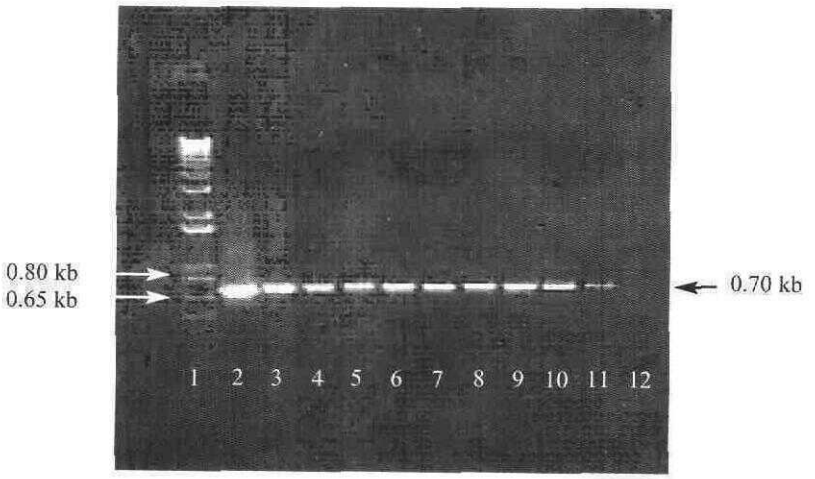


Figure 4. Amplified internal fragment of nptII *cDNA (0.7 kb) from leaves.*
Lane 1: 1 kb Plus Ladder (Gibco-BRL); Lane 2: Positive control (PRIscFv.4715); Lane 3 to 11: Transgenic plants; Lanes 12: Control plant.

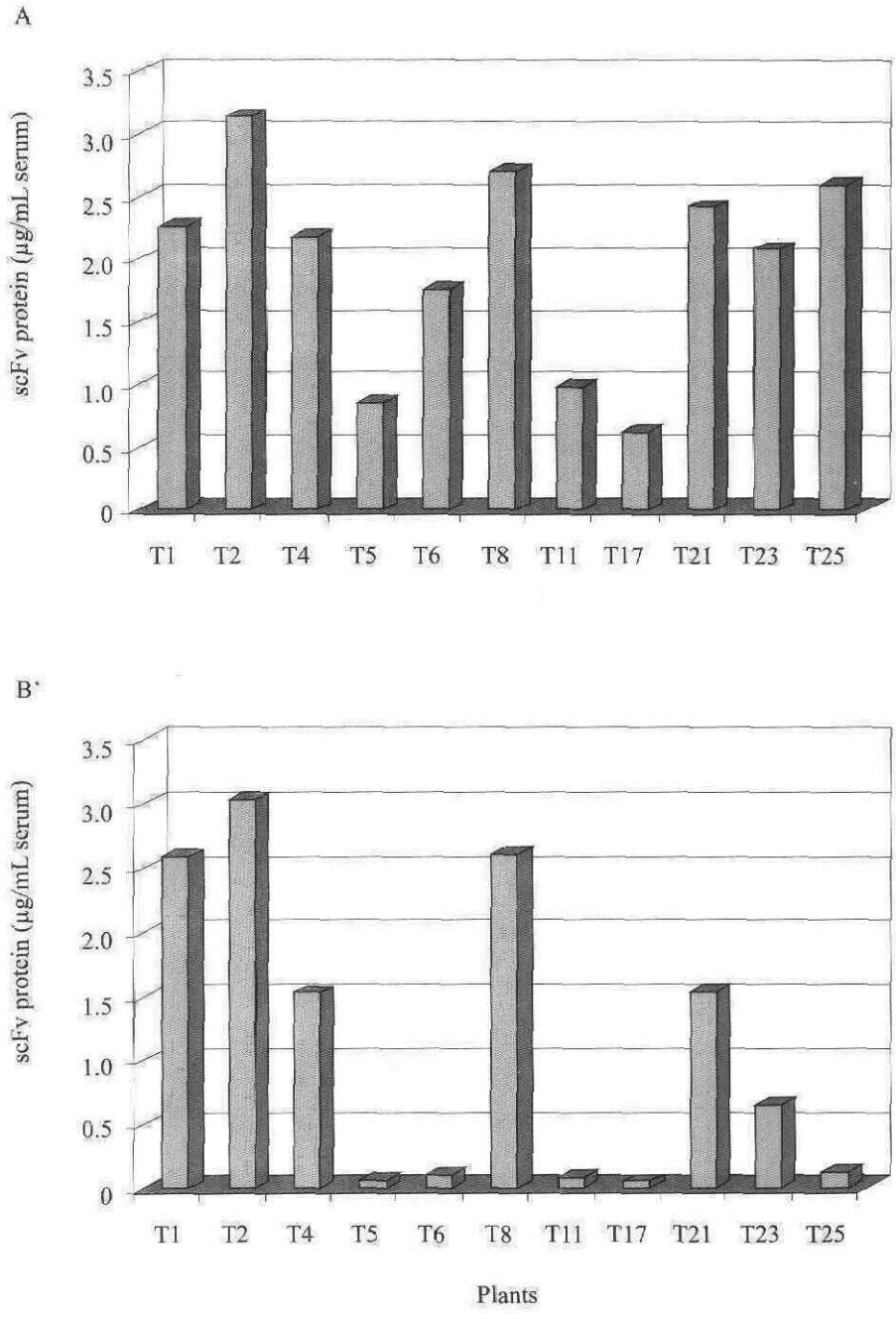


Figure 5. Content of scFv 4715 protein in the latex serum of 11 transgenic plants as determined by ELISA Format 1 *(A) and* Format 2 *(B). Results are the means of three readings. Latex serum from four control plants had <0.04 g/mL scFv 4715 protein for both* Format 1 *and* Format 2. *(Results not presented.)*

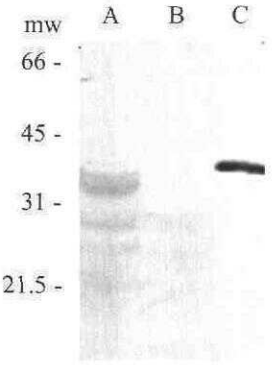


Figure 6. Western blot of recombinant scFv protein following SDS-polyacrylmide gel electrophoresis. Immuno-detection was by the monoclonal antibody against the hydrophil-2 fragment of the recombinant protein. A: Latex extract from transgenic plant; B: Latex extract from untransformed control plant; C: scFv protein positive control.

protein and *Streptococcus* protein, as compared with *Format 1* where the scFv protein was coated on to the ELISA plate directly.

When *Format 2* ELISA was employed, six of the 11 transgenic plants were found to be positive (*Figure 5B*). These results showed that the scFv protein synthesised in the latex of these six plants were functional as antibodies in that they recognised the antigenic protein against which the antibodies were originally developed. The highest reading obtained was 6 μg scFv protein per mL of latex serum. The results also showed that the recombinant proteins synthesised in the latex of the other five plants were functioned poorly as antibodies. It should be noted, nevertheless, that readings from these five plants, even though they were low, were still significantly higher ($p<0.001$) than readings from untransformed control plants. Hence, not only did the amounts of scFv proteins produced vary between the different transformants, the proteins also varied in immunogenecity. The reason for these differences is not clear.

The scFv 4715 content in *Hevea* latex was measured in three transgenic plants at 13–14 months, 16–19 months and 22–23 months after transplanting to soil. An increasing trend in the concentration of the recombinant protein in latex was observed as the plants aged (*Figure 7*).

DISCUSSION

Through genetic transformation, *Hevea brasiliensis* was shown to be able to synthesise a functional single chain variable antibody (scFv) fragment in its latex. This demonstrates that the transgenic rubber tree has the potential to function as a natural, living, bioreactor for the production of pharmaceuticals and other valuable recombinant proteins, of which the functional antibody fragment is an example. Such a production system combines strength of the transgenic animal (continual protein production in the milk) and that of the transgenic plant (low cost of maintenance; simple clonal propagation). In transgenesis,

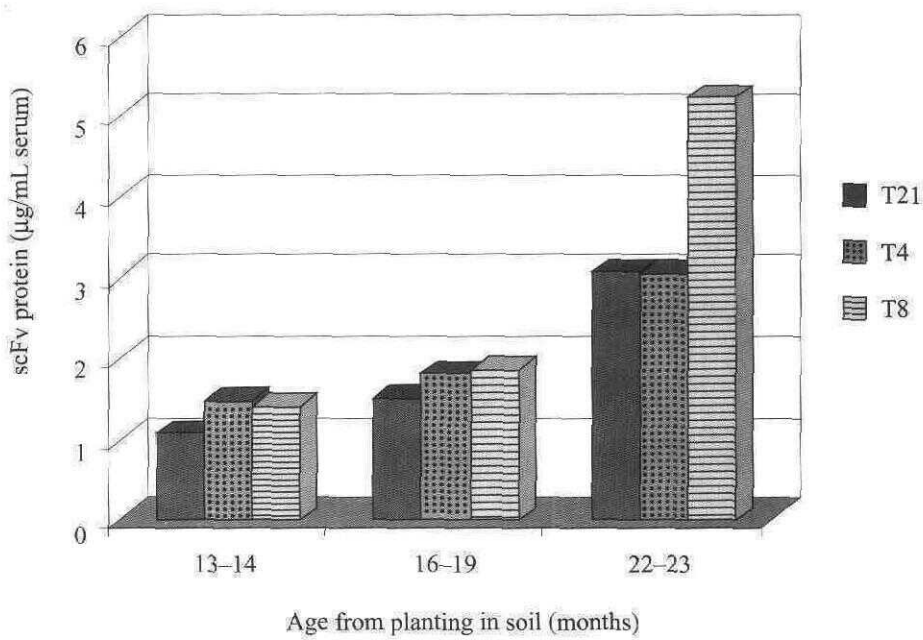


Figure 7. Content of scFv 4715 protein in the latex serum of three transgenic plants (T21, T4, T8) as determined by ELISA Format 2 *at three different ages.*

the most common public perception of hazardous proteins concerns recombinant foods that are unprocessed (*e.g.* fruits) or minimally processed (*e.g.* flour). Unlike transgenic foods, it is unlikely that the scFv protein will be used in an unpurified form. Purification of recombinant protein from rubber tree latex would probably not be any more complex or difficult than comparable procedures employed for recombinant proteins synthesised by micro-organisms in bioreactors.

The scFv protein concentration detected in the latex was about 6 µg/mL latex serum for the most productive plant. This level is probably too low to be of commercial value but it is too soon to draw firm conclusions on the practicality of using the rubber tree for the production of foreign proteins. There are three reasons why an optimistic outlook is not out of place. Firstly, as reported above, there was a very wide variation in productivity levels among individual plants. Hence, it is possible that very high productivity transformants could emerge in the course of further transformation experiments. Secondly, an increasing trend in the recombinant protein concentration was observed when scFv protein readings were taken at different intervals, from 13–22 months. Hence, the productivity of the transgenic plants could reach a level of commercial significance when they are more mature. Thirdly, the upstream DNA sequences of hevein and the rubber elongation factor (REF), which are highly expressed proteins in natural rubber latex, have been cloned recently. The 'universal' CaMV 35S promoter has thus far been employed in the gene constructs used in

Hevea transformation. While this has been shown to be effective in mediating protein expression in the latex[5], it might nevertheless be more advantageous to employ specific promoters and upstream controlling sequences that direct expression of the protein in the latex. Gene constructs used in future *Hevea* transformations would include variants with the CaMV 35S promoter substituted with native *Hevea* promoters that might be more efficient.

Stability of the scFv transformants will be monitored in the future as the plants grow. In this regard, *Hevea* transformed with the *gus* gene in a previous study had continued to express the GUS protein over three successive vegetative generations[5], implying high stability of the inserted gene.

Date of receipt: March 2002
Date of acceptance: September 2002

REFERENCES

1. WALL, R.J., KERR, D.E. AND BONDIOLI, K.R. (1997). Transgenic Dairy Cattle: Genetic Engineering on a Large Scale. *J Dairy Sci.*, **80**, 2213–2224.

2. DANIELL, H., STEPHEN, J., STREATFIELD, J. AND WYCOFF, K. (2001). Medical Molecular Farming: Production of Antibodies, Biopharmaceuticals and Edible Vaccines in Plants. *Trends Pl. Sci.*, **6**, 219–226.

3. YEANG, H.Y., AROKIARAJ, P., JAAFAR, H., HAMZAH, S., ARIJA, M.A.S. AND JONES, H. (1998). Rubber Latex as an Expression System for High-value Proteins, *Engineering Crop Plants for Industrial End Uses (Shewry, P.R., Napier, J.A. and Davis, P.J. eds.)*, pp. 55–64. London: Portland Press.

4. AROKIARAJ, P., JONES, H., HAFSAH JAAFAR, COOMBER, S. AND CHARLWOOD, B.V. (1996) Agrobacterium-mediated Transformation of *Hevea* Anther Calli and Their Regeneration into Plantlets. *J. nat. Rubb. Res.*, **11(2)**, 77–86.

5. AROKIARAJ, P., YEANG, H.Y., CHEONG, K.F., HAMZAH, S., JONES, H., COOMBER, S. AND CHARLWOOD, B.V. (1998) CaMV 35S Promoter Directs β-glucoronidase Expression in the Laticiferous System of Transgenic *Hevea brasiliensis* (rubber tree). *Pl. Cell Rep.*, **17**, 621–625.

6. AROKIARAJ, P., JONES, H., CHEONG, K.F., COOMBER, S., CHARLWOOD, B.V. (1994) Gene Insertion into *Hevea brasiliensis*. *Pl. Cell Rep.*, **13**, 425–431.

7. VAN DER LOGT, C.P.E., SIDEBOTTOM, C.S. AND DAVIS, P.J. (1998) Antibody Production in Plants, *Engineering Crop Plants For Industrial End Uses (Shewry, P.R., Napier, J.A. and Davis, P.J. eds.)*, pp. 17–33. London: Portland Press.

8. HAMILL, J.D., ROUNSLEY, S., SPENCER, A., TODD, G. AND RHODES, M.J.C. (1991) The Use of Polymerase Chain Reaction in Plant Transformation Studies. *Plant Cell Rep.*, **10**, 221–224.

9. CROWTHER, J.R. (1995). *ELISA Theory and Practice*. Totowa, New Jersey: Humana Press.

10. LAEMLLI, U.K. (1970) Cleavage of Structural Proteins During Assembly of the Head of Bacteriophage T4. *Nature*, **277**, 680–685.

11. BOLLAG, D.M., ROZYCK, M.D. AND EDELSTEIN, S.J. (1996) *Protein Methods*. New York: Wiley-Liss, Inc.