Bacterial and plant-produced scFv proteins have similar antigen-binding properties

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Abstract A gene encoding a single-chain variable (scFv) antibody fragment was expressed as a cytoplasmic and endoplasmic reticulum-targeted protein in transgenic tobacco plants. In both cases, the scFv accumulated up to 0.01% of total soluble protein (TSP). The same scFv fragment was also produced in the periplasm of *Escherichia coli*. Measurement of the affinity by ELISA indicates that the affinity of the bacterially made scFv is about 80-fold lower than that of the parental F_{ab} fragment. The results suggest that the affinity of the plant-produced scFv fragments is reduced to a similar extent, implying that all the plant-produced scFv fragments are antigen binding.

Key words: Antibody affinity; *Escherichia coli*; Heterologous gene expression; *Nicotiana tabacum*; Single-chain variable fragment; Transgenic plant

1. Introduction

Antibody expression in plants [1-6] provides a means to interfere with the biological activity of its antigen. In this way, plant cell metabolism can be investigated [7,8] and/or specifically modified to obtain or omit certain products. On the other hand, pathogen-resistant plants can be obtained by expressing antibodies against a protein or other organic molecule necessary for pathogenesis [9,10]. Since many interesting antigen targets are located in the cytoplasm of the plant cell, much research is directed towards the expression of functional antibodies or antibody fragments in this reducing environment. Hiatt et al. [1] demonstrated that for correct assembly of complete antibodies, the individual immunoglobulin chains need to be directed to the endoplasmic reticulum (ER).

In a single-chain variable (scFv) antibody fragment the heavy- and light-chain variable domains are tethered together by a flexible peptide linker. An scFv protein thus retains the antigen-binding potential of a complete antibody or F_{ab} fragment and consists of only one polypeptide chain, avoiding the need for transport to the ER for assembly. So far, the expression of functional scFv fragments in the cytoplasm [7,9], apoplast [11], and ER [8,12] of plant cells has been reported. When the expression of scFv-encoding genes with and without signal peptide sequence was compared, lower or even no accumulation of the cytosol-targeted protein was observed, both in leaves [11,12] and in seeds [13]. This suggests a lower stability of scFv proteins in the cytoplasm. Yet, intracellular expression of an anti-artichoke mottled crinkle virus scFv in *Nicotiana benthamiana* generated plants with scFv accumulation levels up to 0.1% of TSP, which resulted in reduced infection incidence and delayed symptom development [9]. However, few data are available on the efficiency of antigen-binding scFv fragment synthesis and on the affinity of the produced scFv fragments for the cognate antigen.

To investigate the properties of plant-produced scFv molecules in more detail, we expressed a MAK33 scFv-encoding gene as a cytoplasmic and ER-targeted protein in tobacco. The accumulation levels were determined and the antigen-binding properties of the plant-produced scFv fragments were compared to those of a bacterially produced scFv.

2. Materials and methods

2.1. Construction of scFv expression plasmids

The monoclonal IgG1 antibody MAK33 [14] is directed against the human creatine kinase-MM (CK-MM). A MAK33 scFv-encoding sequence was constructed by polymerase chain reaction (PCR) according to [15], using as templates the plasmids pBT111 and pAD1102, containing the cDNA for the light chain and the F_d fragment of the MAK33 antibody, respectively [14]. The resulting sequence encodes an scFv in the configuration V_L-$(Gly_4Ser)_3$-V_H (Fig. 1A). The approximately 750-bp PCR product was gel-purified and subcloned into pGEM2, yielding plasmid pAS1. The DNA sequence of the insert was determined by the dideoxy chain-termination method [16]. The sequence encoding the signal peptide of the 2S2 storage protein of *Arabidopsis thaliana* [17] was fused to the *scFv*-encoding sequence using synthetic oligonucleotides (Fig. 1A). The *scFv*- and *ss-scFv*-encoding sequences were excised and cloned into the P35S-3′*ocs* cassette of the plant expression vector pGV1990 [5], resulting in pAS2 and pSSAS2, respectively (Fig. 1B). For expression in *Escherichia coli*, the *scFv*-encoding sequence in pAS1 was reamplified with specific primers introducing an amino-terminal *Sfi*I site and a carboxy-terminal *Not*I site. By subsequent cloning into the phagemid pHEN1 [18], an exact amino-terminal translational fusion of the *scFv*-encoding sequence with the pelB signal sequence and a carboxy-terminal fusion with the *c-myc* tag sequence was obtained (Fig. 1A). The resulting phagemid was named pBAS2.

2.2. Expression in E. coli

Cultures of *E. coli* HB2151 [18] transformed with the pBAS2 phagemid were grown and periplasm and culture supernatant, containing soluble scFv, were prepared as described in the Expression Module of the RPAS kit (Pharmacia). The TSP concentration in the bacterial extracts was determined with the Bio-Rad protein assay [19].

2.3. Plant transformation and molecular analysis of transgenic plants

The plasmids pAS2 and pSSAS2 were mobilised from *E. coli* to *Agrobacterium tumefaciens* C58C1Rif^R (pGV2260) [20] by triparental

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Abbreviations: EDTA, ethylenediaminetetraacetic acid; ER, endoplasmic reticulum; PCR, polymerase chain reaction; PMSF, phenylmethylsulphonyl fluoride; SDS, sodium dodecyl sulphate; TSP, total soluble protein

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A

scFv DI--(V_L)--LKGGGGSGGGGSGGGGSEV--(V_H)--SS.

ss-scFv MANKLFLVCATFALCFLLTNADI--(V_L)--LKGGGGSGGGGSGGGGSEV--(V_H)--SS.

pelB-scFv MKYLLPTAAAGLLLLAAQPAMADI--(V_L)--LKGGGGSGGGGSGGGGSEV--(V_H)--SSAAAEQKLISEEDLNGAA.

B

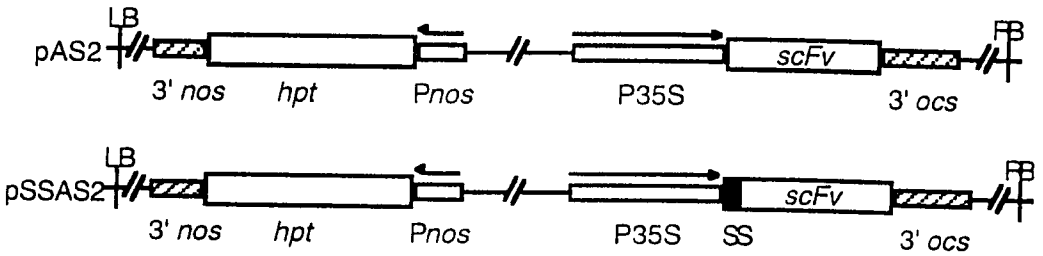

Fig. 1. A: Predicted amino acid sequence of the different scFv-encoding genes expressed in plants and bacteria. The 15-amino-acid linker peptide is presented in grey; the 21-amino-acid 2S2 signal peptide (ss-scFv) and the 22-amino-acid pelB signal peptide (pelB-scFv) are presented in bold; the c-*myc* tag is underlined. The complete MAK33 scFv-encoding sequence can be requested from the authors. B: Outline of the T-DNA constructs for *scFv* (pAS2) and *ss-scFv* (pSSAS2) expression. Abbreviations: P35S, cauliflower mosaic virus promoter; P*nos*, promoter of the nopaline synthase gene; SS, signal peptide of the 2S2 storage protein of *Arabidopsis thaliana*; *hpt*, hygromycin resistance gene; 3'*nos*, 3' end of the nopaline synthase gene; *scFv*, MAK33 single-chain Fv fragment-encoding sequence; RB, right border; LB, left border.

mating. Transformation of *Nicotiana tabacum* SR1 was done by leaf disc infection as described by [21] and transgenic GV-AS and GV-SSAS plants were selected on hygromycin-containing (50 μg/ml) medium.

For comparative protein and RNA analysis, mature leaves were harvested. Part of the leaf was used for protein extraction (see section 2.4), the remaining leaf tissue was frozen in liquid nitrogen, and stored at −70°C for later RNA extraction as described [22]. Total RNA (2 μg) was subjected to electrophoresis in a denaturing 1.2% agarose gel and blotted onto a nylon membrane (Hybond N, Amersham). The scFv mRNA was detected using a riboprobe, radioactively labelled as described in the SP6/T7 transcription kit (Boehringer Mannheim). Hybridisation was carried out in 50% formamide, 5×SSPE, 0.25% milk powder, 0.5% SDS and 20 μg/ml sheared and denatured salmon sperm DNA at 65°C for 16 h. The membranes were washed at 68°C in 3×SSC, 0.1% SDS (15 min, two changes) and in 1×SSC, 0.1% SDS (15 min, two changes).

2.4. ELISA

MAK33-specific antiserum was produced in rabbits using MAK33 IgG/F_{ab} (Poly) complex (Boehringer) as immunogen. IgGs were isolated on protein A-Sepharose CL-4B (Pharmacia). IgGs recognising constant regions of the MAK33 antibody were removed by negative immune adsorption. Therefore, the monoclonal antibody A8 (Boehringer), which contains the same constant domains as the MAK33 antibody, was coupled to CNBr-activated Sepharose. After several passages through a column of A8-Sepharose, the flow-through contained only MAK33 Fv-specific antibodies, as judged by the absence of signal in an A8-coated ELISA.

Transgenic GV-AS and GV-SSAS plants were analysed by ELISA at two developmental stages. Young leaves were harvested from sterile-grown plants (7–8-leaf stage); as mature leaves the fifth big leaf (counting from the top) was taken of greenhouse-grown plants just before flowering (flower buds formed but not yet open). TSPs were extracted from the leaves by homogenisation in 167 mM Tris-HCl pH 8.0, 167 mM EDTA pH 8.0, 1.72 mM PMSF, 630 μM leupeptin (1/3 μl buffer per mg tissue) at 4°C. The extracts were centrifuged at 14900×*g* for 10 min at 4°C and the supernatant was centrifuged again for 10 min for young leaves or for 10 min and 5 min for mature leaves. The TSP concentration in the supernatant was determined with the Bio-Rad protein assay [19] using bovine serum albumin as a standard.

To determine the total amount of scFv protein in the extracts, a sandwich ELISA was set up and performed as described [23]. The rabbit MAK33 Fv-specific antibodies were used as the primary antibody and a derived fraction, biotinylated with the Biotin-labeling kit (Boehringer), was used as the secondary antibody. The secondary antibody was detected with anti-biotin-AP F_{ab} fragments (Boehringer) in ELISAs for the quantification of plant scFv and with streptavidin-AP (Boehringer Mannheim) in ELISAs for the quantification of bacterial scFv.

Antigen binding of the scFv protein in the extracts was checked by an indirect ELISA which was adapted from the MAK33-specific ELISA [5]. Streptavidin-coated wells (Boehringer) were incubated for 1 h at room temperature with 200 μl of a 1 μg/ml biotinylated human CK-MM solution. The MAK33 Fv-specific antibodies were used as the primary antibody and sheep anti-rabbit IgG-AP (Boehringer) as the secondary antibody.

For both ELISAs, the reaction was developed with *p*-nitrophenyl phosphate and the absorbance was kinetically measured at 405 nm using a microtitre plate reader. In all tests, hybridoma-derived MAK33 F_{ab} fragment (Boehringer) was used as a standard. Since detection of antibodies in the ELISAs is specific for the variable regions of the MAK33 antibody, we assumed that a F_{ab} molecule is detected with the same sensitivity as an scFv molecule. The scFv amounts can be calculated on the basis of a standard curve with the MAK33 F_{ab} fragment, taking into account their difference in molecular weight. Extracts of wild-type *N. tabacum* SR1 or periplasm and medium from an *E. coli* culture of HB2151 transformed with the phagemid pHEN1 were used as negative controls.

2.5. Western blotting and immunoprecipitation

SDS-PAGE was performed on mini-gels, essentially as described by [24]. Bacterial proteins were precipitated with deoxycholate/trichlor-

oacetic acid (DOC/TCA) and analysed by enhanced chemiluminescence Western blotting, using polyclonal rabbit anti-MAK33 IgG or the anti-c-*myc* 9E10 monoclonal antibody [25] and horseradish peroxidase-labelled secondary antibody (ECL, Amersham).

Protein extracts from GV-AS and GV-SSAS transgenic plants were obtained by homogenising leaves in 50 mM Tris-HCl (pH 8.0), 50 mM EDTA (pH 8.0), 100 mM NaCl, 2 mM PMSF, 50 μM leupeptin, 5 mM ascorbic acid (5 μl buffer per mg tissue) at 4°C. The extracts were centrifuged at 5000×*g* for 10 min at 4°C and the supernatant was centrifuged again for 5 min. The TSP concentration was determined as mentioned in section 2.4.

The scFv protein was immunoprecipitated from the leaf extracts by incubating (2 h at room temperature) equal amounts of TSP (1750 μg) with biotinylated polyclonal rabbit MAK33 Fv-specific IgG bound to streptavidin-coated Dyna beads (Dynal). The beads were precipitated using a MPC-E magnetic particle concentrator (Dynal). Bound proteins were released by boiling in reducing sample buffer and analysed by Western blotting, essentially as described [26]. Detection was achieved using polyclonal rabbit anti-MAK33 IgG as the primary antibody and sheep anti-rabbit IgG-AP (Boehringer) as the secondary antibody.

2.6. *N-terminal sequencing of the bacterially produced scFv protein*

Periplasmic and culture medium proteins were precipitated with DOC/TCA and resolved by SDS-PAGE. Proteins were electroblotted on Immobilon and N-terminal amino acid sequence analysis of the 32-kDa scFv-specific band was performed as described [27].

2.7. *Affinity determination*

The affinity constants of periplasmic scFv and hybridoma-derived F_{ab} in solution were determined with a competition setting of the indirect ELISA (section 2.4) according to [28]. Samples of periplasmic scFv or hybridoma-derived MAK33 F_{ab} (1.5×10^{-9} M) at a constant concentration were incubated with increasing amounts (final concentrations from 3.7×10^{-10} M to 1.5×10^{-6} M) of human CK-MM (BiosPacific) for 15 h at 25°C. The mixtures were then transferred onto plates which had been precoated with 100 ng biotinylated human CK-MM per well as described for the indirect ELISA. After incubating for 15 min at 25°C, the plates were washed and bound antibody fragments were detected as described in section 2.4. The affinity constants were calculated on the basis of the Klotz equation, which allows the determination of dissociation constants, even when the specific antibody concentration is unknown [28]. As a control, the affinity constant of the F_{ab} fragment was also calculated on the basis of the Scatchard equation [28].

Table 1
scFv accumulation level in leaf tissue of 17 GV-AS and 16 GV-SSAS independent sterile-grown primary transformants

Transformant	ng scFv/mg TSP
GV-AS 8	25 ± 5
GV-AS 6	22 ± 3
GV-AS 28	22 ± 6
GV-AS 5	13
GV-AS 13	13 ± 5
GV AS 18	13 ± 5
GV-AS 27	13
GV-AS 14	12
GV-AS 24	12 ± 6
GV-AS 11	9
GV-AS 15	9
GV-AS 26	9
GV-AS 29	9
GV-AS 1	8
GV-AS 2	8
GV-AS 10	8
GV-AS 21	6
GV-SSAS 16	55 ± 18
GV-SSAS 15	40 ± 21
GV-SSAS 14	22 ± 7
GV-SSAS 19	19 ± 6
GV-SSAS 7	18 ± 8
GV-SSAS 9	14 ± 2
GV-SSAS 2	13
GV-SSAS 3	13
GV-SSAS 13	13
GV-SSAS 20	13 ± 5
GV-SSAS 5	12
GV-SSAS 18	12
GV-SSAS 10	9
GV-SSAS 8	8

When the scFv accumulation level was determined in two independent ELISAs, the mean value (± standard deviation) is given.

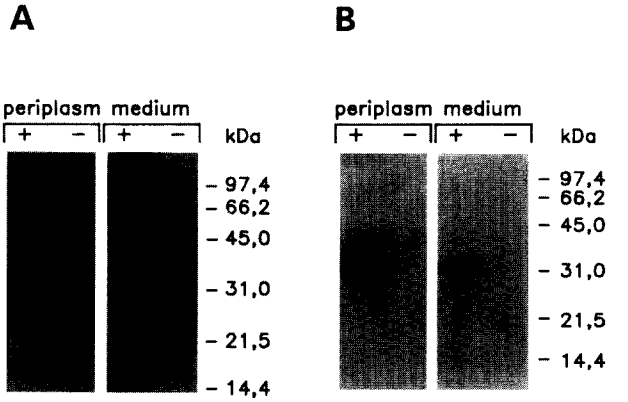

Fig. 2. Immunoblot of periplasmic proteins (10 μg) and culture medium proteins (10 μg) from transformed *E. coli* cells developed with polyclonal rabbit anti-MAK33 IgG (A) and with 9E10 monoclonal antibody (B) as primary antibodies. −, extracts from *E. coli* HB2151 transformed with the phagemid pHEN1; +, extracts from *E. coli* HB2151 transformed with the phagemid pBAS2.

3. Results

To compare the expression of a cytoplasmic and ER-targeted scFv protein in plants, *N. tabacum* was transformed in parallel with the T-DNA vectors pAS2 and pSSAS2 (Fig. 1B). The scFv accumulation was initially determined in young leaves of 30 GV-AS and 19 GV-SSAS-independent transformants. The scFv levels obtained by analysis with the sandwich ELISA are presented in Table 1. From the analysed plants, 13 GV-AS and three GV-SSAS transformants gave no detectable signal, which means that the scFv amount in young leaves of these plants was lower than 6 ng scFv/mg TSP. The scFv accumulation levels in both GV-AS and GV-SSAS transformants are low, between 6 ng and 55 ng scFv/mg TSP. Also in both series of transgenic plants the inter-transformant variability was very low: the difference between the lowest and highest value was only 4-fold for the pAS2 construct and 7-fold for the pSSAS2 construct. This is very probably due to the fact that the detection limit of 6 ng scFv/mg TSP did not allow the analysis of the very low accumulators. Eleven GV-AS and 10 GV-SSAS-independent transformants were grown to maturity and the scFv expression in mature leaves was analysed at protein and mRNA level. Sandwich ELISA demonstrated accumulation levels which were equal to or, for 80% of the analysed plants, 1.5–8-fold higher than the accumulation levels in young leaves. The maximum accumulation level in mature leaves was 0.01% of TSP both for expression in the cytoplasm and targeting to the extracellular compartments of transgenic plants. In contrast with the low protein levels, Northern analysis showed that GV-AS and GV-SSAS plants

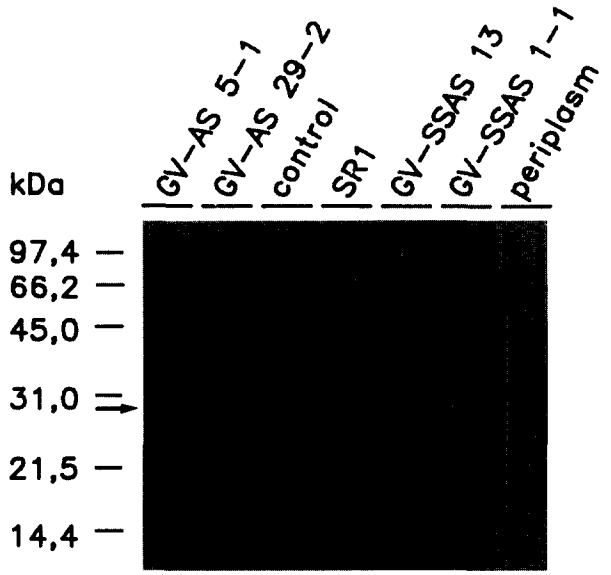

Fig. 3. Immunoblot of scFv immunoprecipitated from different GV-AS and GV-SSAS leaf extracts. The lane marked SR1 is from an untransformed control plant. The lane marked control is from a reconstruction experiment in which bacterial scFv-containing periplasm (10 μg) was added to SR1 leaf extract; it served as a control for the immunoprecipitation. The lane marked periplasm contains periplasmic proteins (10 μg) from *E. coli* HB2151 transformed with the phagemid pBAS2. The position of plant scFv is indicated by an arrow.

produced normal scFv mRNA levels (data not shown), which were as expected for a 35S promoter-regulated gene expression. No correlation was found between scFv mRNA level and the scFv protein level.

The amount of antigen-binding scFv protein in the extracts from young and mature leaves was determined with an indirect ELISA. Only in two GV-AS and two GV-SSAS young leaf extracts a significant signal was obtained; it was calculated that the antigen-binding signal of the accumulated scFv in the GV-AS transformants and in the GV-SSAS transformants was 2.5% and 5%, respectively, of the signal obtained for corresponding amounts of F_{ab} fragment. In the mature leaf extracts from seven GV-AS and four GV-SSAS transformants antigen-binding scFv could be detected. Again the average signal found for cytoplasmic and ER-targeted scFv was only 1% of the antigen-binding signal of equal amounts of F_{ab} protein. These low signals can be caused by the fact that only a small fraction of the accumulated scFv molecules is correctly folded into antigen-binding fragments or they can be a consequence of a reduced antigen affinity of the scFv fragment in comparison to the F_{ab} fragment. Due to the low signal of antigen-binding scFv in tobacco leaf extracts, the affinity of the plant-produced MAK33 scFv could not be determined directly with a competition set-up of the indirect ELISA [28]. Therefore, bacterial expression of the MAK33 scFv-encoding gene was performed for further characterisation of the protein. The scFv accumulation levels, as determined by sandwich ELISA, were about 8 μg/mg TSP in the periplasm and 0.13 μg/ml in the culture medium of transformed *E. coli* cells. The antigen-binding signal in the indirect ELISA of the bacterially produced scFv fragments was also only 4% of the signal obtained for corresponding amounts of F_{ab} fragment. The affinity of the periplasmic MAK33 scFv for its antigen CK-MM was determined and appeared to be approximately 80-fold lower than that of the hybridoma-derived MAK33 F_{ab} fragment (Table 2).

The integrity of the bacterial and plant-produced scFv molecules was checked by immunoblot analysis. Polyclonal rabbit anti-MAK33 IgG revealed a major band of about 32 kDa and a minor band of about 30 kDa both in the periplasm and in the medium of bacterial cultures (Fig. 2A). Western analysis with the anti-c-*myc* 9E10 monoclonal antibody (Fig. 2B), however, only detected the 32-kDa band, indicating that the 30-kDa band represents a minor fraction of scFv protein in which the c-*myc* tag is proteolytically cleaved off, a phenomenon which has been reported before [29,30]. N-terminal protein sequence analysis of the major scFv band showed that the pelB signal peptide was correctly processed. By immunoprecipitation and subsequent Western blotting, we could show an scFv-specific band of about 30 kDa in both GV-AS and GV-SSAS leaf material (Fig. 3). The scFv migrated with a higher apparent molecular weight than its calculated molecular weight of 26 548, as has been found for other scFv proteins with the $(Gly_4Ser)_3$ linker [31,32]; this can be partly explained by the low residue weight of Gly and Ser in the linker. The scFv protein in GV-AS and GV-SSAS leaf extracts is running on the same height, suggesting that the 2S2 signal peptide is cleaved off and that the scFv was indeed translocated to the ER in the GV-SSAS plants.

4. Discussion

In this report the accumulation levels and the antigen-binding properties of bacterial and plant-produced scFv fragments were determined and compared using ELISA measurements. In tobacco leaves, the yield of both the cytosolic and ER-targeted MAK33 scFv protein was very low, the highest level being 0.01% of TSP. Other studies on scFv expression in plants also report low cytoplasmic accumulation [7,12,13]. This can be partly explained by the fact that in the reducing environment of the cytosol the stabilising intramolecular disulphide bridges are not formed efficiently. Indeed, several reports document higher accumulation levels when the scFv fragments were directed towards the oxidising environment of the ER [11-13]. However, another scFv protein appeared to be stable in the cytosol since yields of 0.1% were obtained [9]. Here, we find that the MAK33 scFv expression is very poor, even when secreted. Recently, it has been demonstrated that the amount of scFv increased significantly, up to 100-fold, by KDEL-mediated retention of the protein in the ER. In all cases, the low scFv protein levels were not a consequence of low mRNA levels.

Determination of the affinity revealed an 80-fold lower affi-

Table 2
Affinity constants of periplasmic scFv and hybridoma-derived MAK33 F_{ab} fragment, as determined by indirect ELISA

Antibody	K_D[a]	K_D[b]
MAK33 F_{ab}	$(2.54 \pm 0.35) \times 10^{-10}$ M	$(4.34 \pm 1.04) \times 10^{-10}$ M
Periplasmic scFv		$(3.38 \pm 0.32) \times 10^{-8}$ M

Presented values are the average (± standard deviation) from at least four independent experiments.
[a]Affinities according to [28] deduced from the Scatchard equation.
[b]Affinities according to [28] deduced from the Klotz equation.

nity for the bacterial MAK33 scFv compared to the hybridoma-derived F_{ab} fragment. Such a dramatic reduction in affinity has been reported before [9], but most scFv proteins with the $(Gly_4Ser)_3$ linker have an affinity which is within the same order of magnitude as that of the F_{ab} fragment or complete antibody [30,31,33-35]. It might be that for the MAK33 antibody the antigen-binding site in the V_L-$(Gly_4Ser)_3$-V_H molecule is less stable than in the F_{ab} fragment where it is maintained by the presence of two disulphide-linked constant domains. Also the folding of this particular scFv to its native state might be hampered by the linker.

The low antigen-binding signal of the produced scFv fragments in the indirect ELISA can be explained by the discrepancy in affinity between the F_{ab} fragment and the scFv fragment. Under the conditions and antibody fragment concentrations used in the indirect ELISA, all the added F_{ab} fragment is captured by the antigen on the plate whereas, due to its 80-fold lower affinity, only a small proportion of the added scFv fragments is captured. The antigen-binding signal for the bacterial and plant-produced scFv fragments was within the same range, suggesting that their affinities are probably comparable. If the latter hypothesis is true, this implies that all the plant-produced scFv fragments are antigen binding. Although it is generally stated that scFv fragments bind to their antigens with similar affinities as the parental antibodies [36], we demonstrated that the affinity of an scFv protein may decrease dramatically. Thus, it is important to determine the antigen-binding properties of an scFv molecule, which can be easily performed on *E. coli*-produced proteins, before expressing it in eukaryotic organisms. By altering the linker length and/or variable domain orientation, the affinity of an scFv molecule might be ameliorated [37-39].

Taken together, the reported results on scFv expression in plants suggest that the primary amino acid sequence of an scFv protein might be important for its stability in different subcellular compartments. The main task for the near future is the directed expression of several scFv fragments in different plant species to pinpoint these and other stabilising factors.

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