

Improving scFv antibody expression levels in the plant cytosol

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Abstract Expression of single-chain antibody fragments (scFvs) in the plant cytosol is often cumbersome. It was unexpectedly shown that addition at the C-terminus of the ER retention signal KDEL resulted in significantly improved expression levels. In this report the cytosolic location of the scFv-CK was confirmed, excluding possible mistranslocation to other subcellular compartments. It was shown that expression of several other scFvs was also improved in tobacco protoplasts. In addition expression was improved in transgenic potato. Changing from KDEL to KDEL did not affect the enhanced protein expression level. Addition of the KDEL motif is a simple and straightforward tool to stabilize in planta cytosolic expression of many scFvs.

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Key words: Localization; KDEL retention signal; N-linked glycosylation; Protein expression; Single-chain antibody fragment; Transgenic plant

1. Introduction

The expression of specific antibodies or antibody fragments in plant cells to obtain phenotypic mutants, like resistance against pathogens [1,2] or altered metabolic pathways [3,4], has proved its potential. The use of single-chain Fv fragments (scFvs), consisting of the variable heavy and light chain domains coupled by a linker peptide, is of particular interest since these molecules were functionally expressed in both the secretory pathway and ectopic environments. Even in the reducing environment of the plant cytosol scFvs can fold into functional molecules. This folding capability has also been demonstrated in *Xenopus* oocytes, COS cells and human cells [5,6].

Despite these successes, cytosolic expression of scFv antibodies in plants is often low or absent [7–11]. If the stability in

the cytosol is an intrinsic property of the variable domains it will be a tedious job to improve expression levels for each individual scFv antibody without losing affinity. It would therefore be more convenient to search for a general approach to stabilize cytosolic scFv molecules without modifying the variable domains.

Recently, we obtained a significantly improved stability in tobacco when the tetrapeptide KDEL was added at the C-terminal end of an anti-cutinase scFv (scFv-CK) designed to be located in the cytosol [9]. This tetrapeptide is the signal for retaining proteins in the endoplasmic reticulum (ER) when translocated into the secretory pathway [3,12–14]. The addition of a four amino acid extension may be a valuable approach to stabilize cytosolic scFvs without modifying the variable domains.

In this report we first established that scFv-CK was present in the cytosol of tobacco, to exclude possible mistranslocation to the ER, reported by [15], as a cause of improved scFv protein expression levels. We then investigated if the tetrapeptide KDEL could also be beneficial for the cytosolic expression level of several other scFv antibodies and be successfully applied for the expression of scFvs in another plant species, potato. Finally, to study whether or not the tetrapeptide could be slightly modified without losing its positive effect on protein stability in the cytosol, the leucine of the tetrapeptide KDEL of one of the scFvs was changed into isoleucine and this antibody fragment was expressed in potato.

2. Materials and methods

2.1. Cell lines, vectors and strains

Single-chain antibodies were constructed starting from the hybridoma cell lines MGR48, MGR49 and MGR59 [16], all producing monoclonal antibodies reacting with β -1,4-endoglucanase, and from the hybridoma line anti-GUS, which produces an anti- β -glucuronidase monoclonal antibody.

For construction of the various 21C5 anti-cutinase scFv genes the vectors pNEM-scFv, pNEM-scFv-K and pCPO-scFv-CK [9] were used.

For transient expression assays scFv antibodies were cloned in pRAP-scFv-SK, pTR2-scFv-SK or pTR2-scFv-S. To obtain pRAP-scFv-SK the vector pUCAP35S [17] was provided with a kappa signal peptide [18] and the scFv cassette with carboxy-terminal KDEL extensions, obtained from the vector pNEM-scFv-K [9]. The vector pRAP-scFv-SK thus obtained can accept other scFv encoding genes as *SalI/NotI* fragments between the kappa signal peptide and the c-myc tag plus C-terminal KDEL extension. To obtain pTR2-scFv-SK and pTR2-scFv-S we first cloned the 1.4 kbp *HindIII* fragment from pCPO5 [19] containing the 35S terminator (T35S), the TR2'-1' dual promoter and octopine synthase terminator (Tocs) into the *HindIII* site of pAP [17]. Then the original *SalI* site, present between the TR1' promoter and Tocs, was removed by filling in, creating the vector

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Abbreviations: EDTA, ethylenediaminetetraacetic acid; ER, endoplasmic reticulum; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid); NP-40, nonidet P-40; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PVDF, polyvinylidene difluoride; rER, rough endoplasmic reticulum; SDS, sodium dodecyl sulfate

The sequences reported in this work have been deposited in the GenBank database (accession numbers AF004403; AF004404; AF004405; AF004406; AF004407).

pTR2. The anti-cutinase scFv cassettes with the coding sequence for signal peptide and C-terminal c-myc tag, with and without KDEL retention signal, were isolated from pNEM-scFv-K and pNEM-scFv as *NcoI/BclI* fragments and inserted into *NcoI/BamHI* digested pTR2, resulting in the vectors pTR2-scFv-SK and pTR2-scFv-S, respectively.

2.2. Construction of scFv genes with coding sequence for glycosylation in the linker

To modify the 202' derived peptide linker [20] of the 21C5 anti-cutinase scFv the V_L domain was reamplified using the primers L5d (5'-GGTGTGCGACGGTGATGTTKTGATGACCCAAA-3') and Vkglyc (5'-AGCCGGATCCGTTGGATTACCTCGAGTTTAT-TTCCARCTTKGTSCC-3'). Restriction sites in the primers, used in the cloning procedure, are underlined. PCR conditions were used as described by [9]. The amplified fragment was inserted into the *SallI/BamHI* restricted vector pNEM-scFv. The resulting pNEM-scFv^{glyc} contained a linker flanked by *XhoI* sites. With respect to the original scFv the Arg at position 1 of the peptide linker was replaced by Leu (Fig. 1). The Ser at position 6 was replaced by Asn, thereby creating a consensus site for N-linked glycosylation [21]. A control scFv construct in which the Asn was replaced by Ser, but the Leu at position 1 retained, was obtained by inserting an *XhoI/BamHI* adapter fragment (5'-TCGAGGGTAAATCCTCCG-3' and 5'-GATCCGAGGATT-TACCC-3') into *XhoI/BamHI* digested pNEM-scFv^{glyc}, creating pNEM-scFv^{Δglyc}. The modified scFv constructs were cloned as *SallI/NotI* fragments from pNEM-scFv^{glyc} and pNEM-scFv^{Δglyc} into pRAP-scFv-SK, creating pRAP-scFv^{glyc}-SK and pNEM-scFv^{Δglyc}-SK, respectively (Fig. 1).

The scFv gene insert from the vector pRAP-scFv-SK was replaced by the *NcoI/NotI* scFv insert from pCPO-scFv-CK, resulting in the vector pRAP-scFv-CK. To obtain scFv-CK constructs with identical linker peptide coding sequences with and without glycosylation signal the *PstI* fragment in this vector was replaced by the same insert from the vectors pRAP-scFv^{glyc}-SK and pRAP-scFv^{Δglyc}-SK. The resulting constructs were verified on proper orientation and subsequently called pRAP-scFv^{glyc}-CK and pRAP-scFv^{Δglyc}-CK, respectively (Fig. 1).

2.3. Immunocytolocalization

Young leaves (length approximately 3 cm) of in vitro grown transgenic tobacco plants, expressing the anti-cutinase scFv-CK and scFv-SK antibodies, were submersed in a solution of 4% formaldehyde, 0.1 M HEPES-NaOH pH 7.5 and 1.5 mM $CaCl_2$. The leaf tissue was cut into strips of approximately 1 × 5 mm, which were degassed briefly in vacuo and left to fix overnight at room temperature. Following washing in water, the leaf strips were dehydrated in ethanol and embedded in LR-Gold resin following standard low-temperature procedures [22]. Immunogold labeling of thin sections was performed essentially as described by [16], using monoclonal antibody 9E10 to the c-myc tag at a concentration of 1 µg/ml as the primary antibody, and a 10 nm goat anti-mouse gold conjugate (Aurion, Wageningen, The Netherlands) for detection. Control labeling experiments were performed with leaf tissue from untransformed plants, and by omitting the primary antibody in the labeling procedure.

2.4. Construction of scFv antibody genes

Isolation of poly(A)⁺ RNA from hybridoma cells was performed using the QuickPrep Micro mRNA purification kit (Pharmacia). cDNA was synthesized using the Pharmacia First Strand cDNA Kit.

To create the scFv₄₈ gene the MGR48 cDNA was amplified by PCR using the primer combination L5d-Nco (5'-CGTGCCATG-GATGTTKTGATGACCCAAACTC-3') and 202VL3 (5'-GGATT-CAGATCCGGATCCTGAGGACTTACCCTCGAGCTTTATTTCC-AGCTTGGTCCC-3') for the V_L domain and 202VH5 (5'-TCAGGATCCGGATCTGAATCCAAAGTCTCAGTCTCAGGTC-AGTTGGTACAGTCTG-3') and VH33b (5'-GCACGTTAAC-CCCGGGTGTGTTTGGCTGCAGAGACAG-3') for the V_H domain. The *NcoI/BamHI* digested V_L and *BamHI/HpaI* digested V_H fragments were ligated together in the *NcoI/SmaI* digested pTR2-scFv-S and pTR2-scFv-SK.

For the scFv₄₉ construction we amplified MGR49 cDNA using primer combination L5h (5'-GGTGTGCGACGGTGACATCCAGAT-GACMCAGWCTMCM-3') and 3KGS to obtain V_L and 5HGS (5'-GGTGGAGGATCCGGTGGAGGAGGTTCTGAGGTYCAGC-TG-CARSA-3') and VH34 (5'-ATGCGTTAACCGTTGTTTGG-CTGMRGAGACDGTGAS-3') to obtain V_H . The V_L and V_H do-

main were fused using splicing by overlap extension (SOE) [23] and initially ligated as a *SallI/HpaI* fragment into pNEM6 [24]. Finally the scFv₄₉ fragment was cloned as *NcoI/NotI* fragment into *NcoI/NotI* digested pTR2-scFv-S and pTR2-scFv-SK.

To create scFv₅₉ MGR59 cDNA was amplified with the primers L5d-Nco and 3KGS2 (5'-CGCCTCCGGAGCCTCCACCACCGGAA-CCACCACCACCGGATCCCCCTTTTATT-TCCARCTTKTGTC-CC-3') for the V_L domain and gsH5c (5'-GGTGGAGGCTCCG-GAG-GCGGAGGATCCGAGGTCCAGCTGCAACARTC-3') and VH33a (5'-GCACGTTACC-CCGGGTGTGTTTGGCTGAGGA-GACKG-3') for the V_H domain. After *NcoI/BspEI* and *BspEI/SmaI* digestion the domains were ligated together in the *NcoI/SmaI* digested pTR2-scFv-S and pTR2-scFv-SK.

To create scFv_{GUS} anti-GUS cDNA was amplified using the primer combination L5h and VLK2 (5'-GACTCGAGTTTGGATTCCGGA-GCCGGATCCTGAGGATTTACCCTCCGTTTATTTCCARCT-TKGTCC-CMG-3') to obtain V_L and primer combination FVH3 (5'-TCAGGATCTGGCTCCGAATCCAAACTCGAGTCTGAGGT-GAAGCTGGTGGARTCTG-3') and VH33 to obtain V_H . The V_L and V_H domains were fused by SOE and initially ligated as a *SallI/SmaI* fragment into pNEM5 [9]. Then the scFv_{GUS} gene was ligated as *NcoI/NotI* fragment into *NcoI/NotI* digested pRAP-scFv-S and pRAP-scFv-SK.

Cloning procedures were according to [25]. All constructs were checked by sequencing [26].

2.5. Transient expression and protein analysis

Transient expression assays in tobacco (*Nicotiana tabacum* cv. Sam-sun NN) leaf protoplasts were performed essentially according to the polyethylene glycol procedure as described by [27]. In case of tunicamycin treatment the transfected protoplast were incubated in TEX medium supplemented with 10 µg/ml tunicamycin [28].

The protoplasts were separated from the incubation medium and lysed by adding an equal volume of 10 mM Tris-HCl, pH 8.0, 1 mM EDTA and 1 mM Pefabloc SC (Boehringer), and subsequent vortexing. The chloroplasts were pelleted by centrifugation at 13 000 × g for 2 min and the supernatant was used for further analysis. For Western analysis one third volume of 4 × SDS-PAGE sample buffer, containing 244 mM Tris-HCl, pH 6.8, 8% (w/v) SDS, 50% (w/v) glycerol and 1 mM Pefabloc SC (Boehringer), was added to an aliquot of the supernatant, equaling 3 × 10⁴ protoplasts. The samples were incubated at 100°C for 5 min and bromophenol blue was added to a final concentration of 0.008% (w/v).

For deglycosylation proteins isolated from 8 × 10⁴ protoplasts in a final volume of 20 µl were denatured by adding 2.2 µl 5% (w/v) SDS, 2% (v/v) β-mercaptoethanol and incubation at 100°C for 5 min. The sample was split into two portions. Both aliquots received 2 µl 0.5 M sodium phosphate, pH 7.5, 3 µl water and 2 µl 10% (v/v) NP-40. To the first aliquot 2 µl PNGase F (500 units/µl, New England Biolabs) was added. As a control, 2 µl water was added to the second aliquot. The samples were incubated at 37°C for 1 h and bromophenol blue was added to a final concentration of 0.008% (w/v).

Protein samples were loaded on a 13% SDS-polyacrylamide gel [29] (Bio-Rad mini protean system). After electrophoresis the proteins were transferred to a PVDF membrane (Millipore) by electroblotting. For immunodetection the membranes were incubated with 1:1000 diluted 9E10 monoclonal antibody [30], followed by a 1:5000 diluted rat anti-mouse alkaline phosphatase conjugate (Jackson Immuno Research). The blots were stained in 0.1 M ethanolamine-HCl pH 9.6, supplemented with 4 mM $MgCl_2$, 5-bromo-4-chloro-3-indolyl phosphate (0.06 mg/ml) and nitroblue tetrazolium (0.1 mg/ml). The relative molecular weights of the proteins were estimated with pre-stained low range molecular weight markers (Bio-Rad).

2.6. Potato transformation and protein analysis

The scFv₄₈ expression cassettes cloned in the transient expression vector pTR2 were isolated as *PacI*-*AseI* fragments and transferred to the binary vector pBINPLUS as described by [17]. The resulting plasmids were introduced into *Agrobacterium tumefaciens* strain AGL0 [31]. This strain was used for the transformation of internodal stem sections of *Solanum tuberosum*, dihaploid genotype 6487-9 (2n = 2x = 24), as described by [32]. Genotype 6487-9 resulted from a cross between genotype 1024-2 [33] and genotype 91-6222-40, a self-incompatible Gg clone selected in 1992 from the material of [34]. Kanamycin resistant transformants were regenerated and total soluble

proteins were extracted by grinding roots essentially as described by [18]. The proteins were analyzed by loading 30 µg total soluble protein homogenate on an SDS-polyacrylamide gel followed by electroblotting and immunodetection as described.

3. Results

3.1. Subcellular location of the scFv antibodies in plant cells

To study possible ‘mistranslocation’ of scFv-CK to the ER we used N-linked glycosylation as a biochemical marker. A mutant, scFv^{glyc}-CK (Fig. 1) was engineered, having a consensus glycosylation site (Asn-Gly-Ser) in the linker peptide. Both this construct and the construct having the original Ser-Gly-Ser peptide coding sequence in the linker (scFv^{Aglyc}-CK, Fig. 1) were expressed transiently in tobacco protoplasts. As controls we took the scFv^{glyc}-SK and scFv^{Aglyc}-SK constructs which carry both the ER translocation signal sequence and an ER retention signal KDEL (Fig. 1). Glycosylation was determined by the relative migration of these proteins on Western blot (Fig. 2). ScFv^{glyc}-SK expression resulted in proteins migrating at 32, 33 and 67 kDa (Fig. 2, lane 1). The 67 kDa protein is thought to be an scFv dimer due to disulfide bridge formation caused by the cysteine present in the linker peptide (Fig. 1) [9]. Due to the presence of β-mercaptoethanol in the reaction buffer this dimer was absent after glycosidase F treatment. When the transfected protoplasts were incubated in the presence of the glycosylation inhibitor tunicamycin or after glycosidase F treatment of the total protein homogenate, the 33 kDa band disappeared and the 67 kDa band slightly shifted to 65 kDa (Fig. 2, lane 2). Expression of scFv^{Aglyc}-SK resulted in 32 and 65 kDa bands which were unaffected by glycosidase F treatment of the total protein homogenate or tunicamycin treatment of the transfected protoplasts (Fig. 2, lanes 3 and 4). This indicates that the 33 kDa and 67 kDa proteins are glycosylated forms of the 32 kDa scFv monomer and 65 kDa scFv dimer. The band intensities of the 33 and 32 kDa bands indicate that approximately 70% of the scFv^{glyc}-SK protein became glycosylated. Because of a three amino acid extension at the N-terminus the apparent molecular

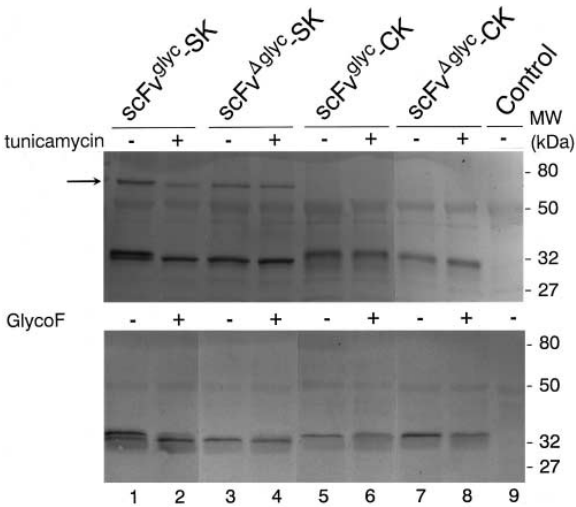


Fig. 2. Determining the glycosylation state of scFv^{glyc}-SK and scFv^{glyc}-CK proteins. Top panel: Western blot of total protein from tobacco protoplasts, transfected with the vectors containing the scFv^{glyc}-SK, scFv^{Aglyc}-SK, scFv^{glyc}-CK and scFv^{Aglyc}-CK gene cassettes, were incubated in the absence (–) or presence (+) of tunicamycin and analyzed on Western blot. Arrow indicates the 67/65 kDa protein bands. Bottom panel: Western blot of total protein from protoplasts, transfected with the vectors containing the scFv^{glyc}-SK, scFv^{Aglyc}-SK, scFv^{glyc}-CK and scFv^{Aglyc}-CK gene cassettes, which had been incubated in the absence of tunicamycin and were either mock treated (–) or treated (+) with the endoglycosidase glycoF. As a negative control (Control) a vector without scFv gene cassette was used.

weight of the expressed scFv^{glyc}-CK and scFv^{Aglyc}-CK proteins is slightly higher than the scFv^{Aglyc}-SK and unglycosylated scFv^{glyc}-SK proteins and was estimated at 33 kDa (Fig. 2, lanes 5 and 7). For both scFv^{glyc}-CK and scFv^{Aglyc}-CK the mobility was unaffected by glycosidase F treatment of the total protein homogenate or tunicamycin treatment of the transfected protoplasts (Fig. 2, lanes 6 and 8). Therefore, we conclude that the scFv^{glyc}-CK protein is not glycosylated, indicating that the scFv-CK antibody, in contrast to the scFv-SK antibody, is not translocated into the ER.

To obtain ‘visual’ proof of the presence of the scFv-CK antibodies in the cytosol, we examined leaf sections of transgenic plants expressing the anti-cutinase scFv-CK and scFv-SK antibodies with immunoelectron microscopy (Fig. 3). We had obtained transgenic plants with maximum expression levels of 0.2% and 1% of total soluble protein for scFv-CK and scFv-SK, respectively [9]. Leaf sections were prepared from transgenic plants expressing scFv-CK at a level of 0.2% and scFv-SK at levels of 0.2% and 1% of total soluble protein. The ultrathin sections of leaves expressing scFv-CK showed gold particles evenly distributed in the cytosol (Fig. 3A,B), indicating the presence of scFvs in that compartment. No gold particles were found in other subcellular compartments. The sec-

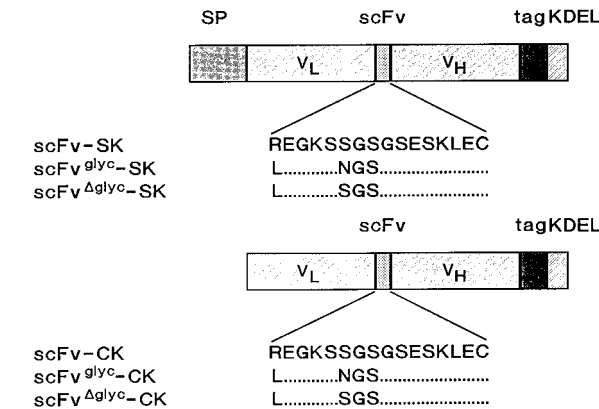


Fig. 1. Diagram of the scFv-SK and scFv-CK constructs with modifications in the linker peptide. The signal for N-linked glycosylation, Asn-X-Ser, was introduced by changing the Ser of both scFv^{Aglyc}-SK and scFv^{Aglyc}-CK to Asn, resulting in the sequence Asn-Gly-Ser in the constructs scFv^{glyc}-SK and scFv^{glyc}-CK, respectively. The N-terminal signal peptide (SP), the single-chain antibody construct (scFv) with the variable light (V_L) and heavy (V_H) chain domains connected by the linker peptide, the C-terminal c-myc tag (tag) followed by the KDEL peptide sequence are indicated.

Table 1 Amino acid sequences of the linker peptides present in the various scFv antibodies	
Single-chain	Linker peptide
scFv ₄₈	-LEGKSSGSGSESKLES-
scFv ₄₉	-(GGGGG) ₃ -
scFv ₅₉	-G ₂ S-(GGGGG) ₃ -
scFv _{CUS}	-REGKSSGSGSESKLES-

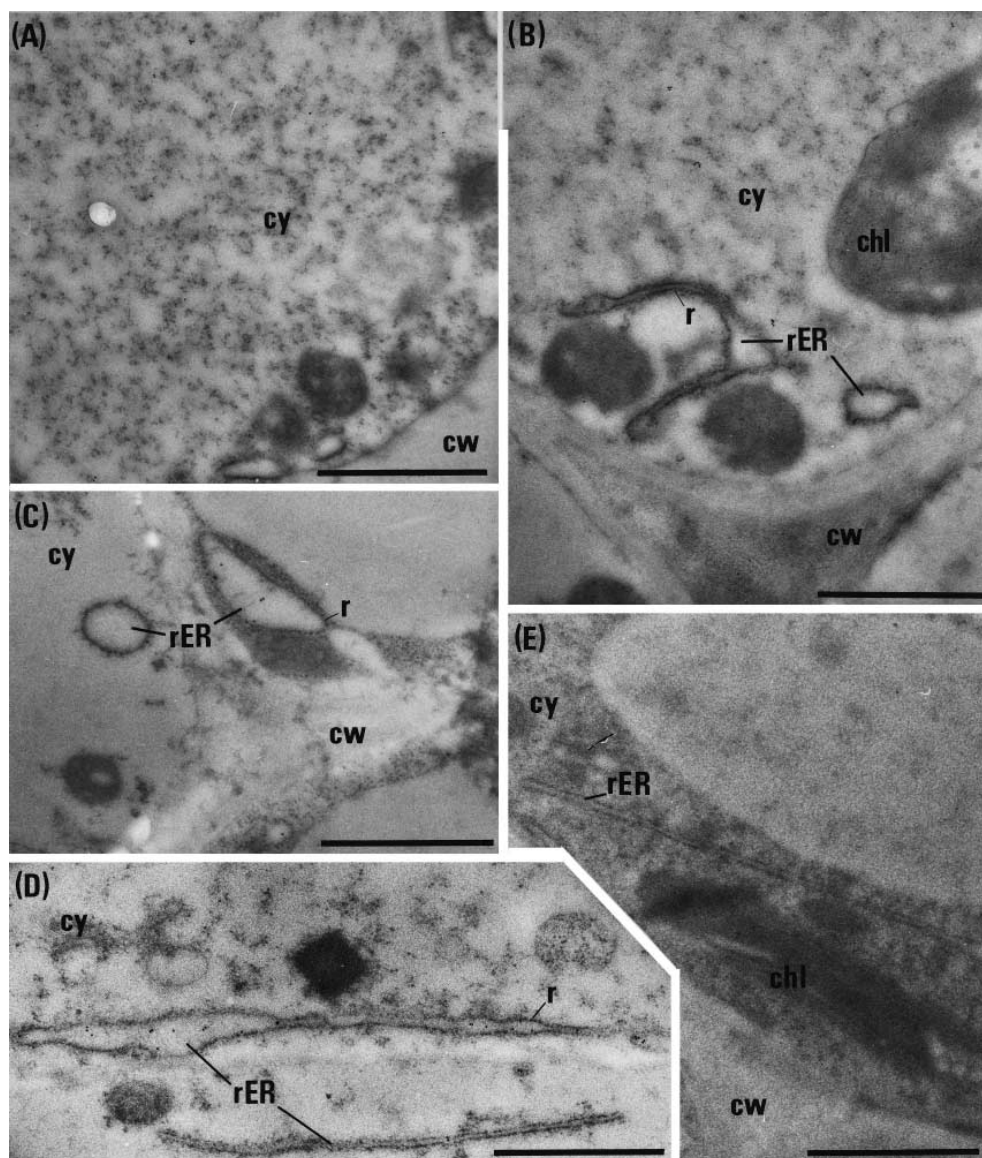


Fig. 3. Localization of scFv-CK and scFv-SK in transgenic tobacco by immunogold labeling and transmission electron microscopy. Leaf sections from plants expressing scFv-CK at a level of 0.2% (A and B) and scFv-SK at a level of 0.2% (C) and 1% (D) of total soluble protein. E: Leaf sections from untransformed plants. chl, chloroplast; cw, cell wall; cy, cytoplasm; rER, rough endoplasmic reticulum; r, ribosome. Bars = 1 μ m.

tions of the leaves expressing the ER-targeted scFv-SK showed gold particles localized in the membranous elements coated with electron dense ribosomes, the rough ER (rER) (Fig. 3C,D). The number of gold particles correlated with the scFv-SK expression level. In several plant cells the structure of some of the rER was not elongated but vacuolated as has been described [35]. No significant labeling was found in untransformed leaf sections (Fig. 3E). We therefore conclude that, as intended, scFv-SK and scFv-CK were located in the ER and the cytosol, respectively.

3.2. Expression of different scFv antibodies in the cytosol of tobacco protoplasts

To determine if the C-terminal KDEL extension could also improve cytosolic expression levels of other scFv antibodies the variable light (V_L) and heavy (V_H) domains of four other antibodies were amplified by PCR and coupled by a synthetic

linker sequence in a 5'- V_L -linker- V_H -3' orientation. These antibodies were MGR48, MGR49 and MGR59, all reacting

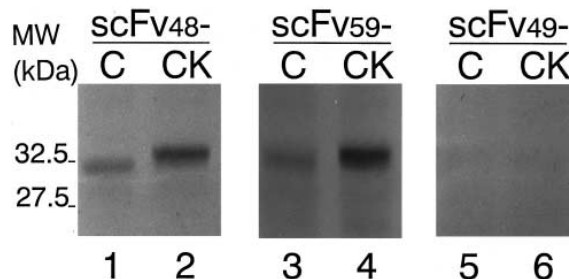


Fig. 4. Western blot analysis of a transient expression assay in tobacco protoplasts transformed with the scFv₄₈, scFv₅₉ and scFv₄₉ constructs without (C) and with (CK) the tetrapeptide KDEL extension.

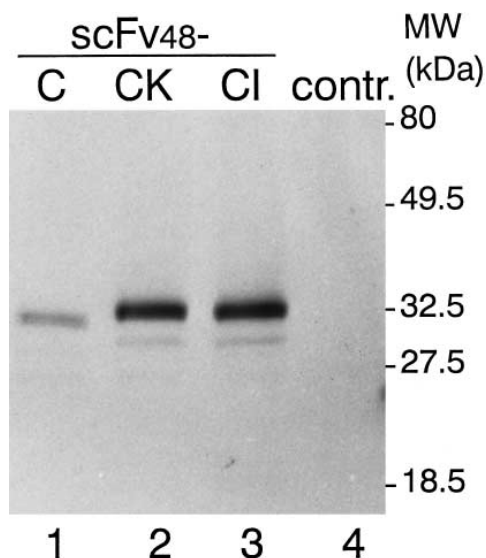


Fig. 5. Western blot analysis of transgenic potato plants expressing scFv₄₈ without (C) and with (-CK) tetrapeptide KDEL extension or with tetrapeptide KDEL extension (CI).

with β -1,4-endoglucanase, and anti-GUS, reacting with β -glucuronidase. The linker sequences coded for 202' [20] and (Gly₄Ser)₃ peptides or derivatives (Table 1). The scFv sequences were directly preceded by the ATG translational start codon and fused in frame with the c-myc tag coding sequence, enabling detection ('C' constructs). The 'CK' constructs carried the additional KDEL coding sequence at the 3'-end.

These scFv constructs were transiently expressed in tobacco protoplasts. The 21C5 anti-cutinase scFv-C and scFv-CK constructs were used as reference. Western blot analysis showed that cytosolic expression of the scFv₄₈ and scFv₅₉ constructs was improved when the KDEL tetrapeptide was present (Fig. 4). The estimated expression levels of scFv₄₈-C and scFv₄₈-CK were 0.02% and 0.06% of total soluble protein, respectively (Fig. 4, lanes 1 and 2). The estimated expression levels of scFv₅₉-C and scFv₅₉-CK were 0.02% and 0.1% of total soluble protein, respectively (Fig. 4, lanes 3 and 4). Poor expression was found with scFv₄₉ for both the C and CK versions. Transient expression of both scFv_{GUS} versions gave no detectable expression (not shown). Apparently, the KDEL tetrapeptide is not capable of improving cytosolic expression of every scFv antibody.

3.3. Cytosolic expression of scFv₄₈ gene cassettes in transformed potato

To determine whether the KDEL extension could improve scFv protein expression in the cytosol of another plant species we transformed C and CK gene constructs of scFv₄₈ to potato by *Agrobacterium* mediated transformation. Independent kanamycin resistant transformants were screened by immunoblotting of total protein extracts (Fig. 5). Only 20% of the scFv₄₈-C transgenics screened showed expression, at an estimated level of 0.03% of total soluble protein (Fig. 5, lane 1). In contrast, 60% of the scFv₄₈-CK transgenics screened showed an expression level estimated at 0.15–0.3% of total soluble protein (Fig. 5, lane 2). This demonstrates that the stabilizing effect of the tetrapeptide in cytosolic expression is not restricted to tobacco.

To study the influence of a modification of the KDEL ex-

tension on protein expression levels, an scFv₄₈-CI gene construct, carrying the KDEI instead of KDEL coding sequence, was introduced into potato. This single amino acid substitution in the tetrapeptide is known to disrupt the retention signal and the protein is no longer retained in the ER [13]. In 80% of the transgenics screened the scFv₄₈-CI protein expression level was estimated at 0.3% of total protein (Fig. 5, lane 3), indicating that the isoleucine replacement did not result in a loss of the stabilizing effect.

4. Discussion

The expression of antibodies in plants is a promising approach for obtaining pathogen resistance or altering metabolic pathways. Success depends on proper targeting to the desired subcellular compartment combined with proper folding and sufficient stability in order to obtain functional antibodies. The recent successes [1,3,4] suggest that scFv antibodies, provided with the proper translocation signals, have these abilities and can even be expressed in the reducing environment of the cytosol. However, these scFv antibodies were never properly localized and cytosolic scFv expression in plants was mostly low or absent. We demonstrated previously that the expression level of an anti-cutinase scFv antibody targeted to the cytosol was significantly enhanced when carrying the KDEL sequence C-terminally (scFv-CK) [9]. Stabilizing scFv antibodies in the plant cytosol by adding this four amino acid sequence, known as the ER retention signal [12], would open new possibilities, but the actual subcellular location was uncertain. Noteworthy in this respect is that for a cytosolic scFv antibody in HeLa cells mistranslocation to the ER was suggested [15]. Furthermore, alternative translocation pathways to the ER of proteins lacking a signal sequence have been described [36–38].

In this paper we located unambiguously the scFv-CK in the cytosol, excluding possible mistranslocation or alternative translocation events to the ER. This was proved using N-linked glycosylation as a biochemical marker and immunoelectron microscopy. N-linked glycosylation is a post-translational modification of proteins and confined to the ER [39]. Transient expression assays in tobacco protoplasts of the positive control, an scFv with ER translocation signal and KDEL extension carrying the glycosylation signal (scFv^{glyc}-SK), clearly demonstrated that glycosylation occurred, indicating that this scFv antibody had been translocated into the ER. However, only 70% of the scFv^{glyc}-SK became glycosylated. This could have been caused by the fact that the ER translocation signal was inefficient, and the scFv^{glyc}-SK was only partially translocated into the ER, or that N-linked glycosylation in the ER was incomplete. The first reason is most unlikely since immunoelectron microscopy showed no scFv-SK outside the ER of the plant cells. Incomplete glycosylation in the ER is more likely. Competition between protein folding and N-linked glycosylation has been described extensively [40–42] and, apart from the X as a determinant in glycosylation efficiency [43], not all the Asn-X-Ser/Thr sequons in a protein become glycosylated [44]. Since the V_L domain can fold independently into a stable domain [45,46] the nascent V_L domain may rapidly begin with the formation of the proper domain structure the moment it is translocated into the ER, making it difficult for glycosyl transferase to attain the glycosylation signal in the linker peptide.

As reported before, the anti-cutinase scFv designed to be located in the cytosol could only be detected when the tetrapeptide KDEL was added C-terminally [9]. We postulated that stabilization may have been caused by mistranslocation into the ER. In this case, all the scFv-CK antibody present would be located in the ER. However, the results showed that scFv^{glyc}-CK was not glycosylated, demonstrating that no mistranslocation into the ER had occurred. The actual subcellular location was confirmed by immunoelectron microscopy of tobacco leaf sections. The scFv-SK was detected in the ER and the scFv-CK in the cytosol. No labeling was found in any other subcellular compartment. When two plants with similar expression levels are analyzed the scFv-CK antibody obviously becomes more diluted, since the cytosol, compared to the ER, is rather large in size. However, the immunodetection on the untransformed control plant showed no labeling. It can therefore be concluded that the four amino acid extension KDEL enhances the anti-cutinase scFv antibody expression levels in the cytosol.

Other scFv antibodies were constructed to investigate a broad applicability of the KDEL extension for stabilizing scFv antibodies expressed in the cytosol. Expression levels were not improved for one anti- β -1,4-endoglucanase scFv (scFv₄₉-CK) and the anti- β -glucuronidase scFv (scFv_{GUS}-CK). However, the other two anti- β -1,4-endoglucanase scFvs (scFv₄₈-CK and scFv₅₉-CK) showed a significantly enhanced protein expression level. This was found in both transient expression assays and stable transformants. As was already demonstrated previously [9] for cytosolic scFvs, the protein expression levels in transient expression assays with tobacco protoplasts are positively correlated with the levels obtained in transformed plants, even if this is a different species. Furthermore, the scFv₄₈-CK protein expression level in the transgenic plants, reaching 0.3% of total soluble protein, is very similar to what was found for the anti-cutinase scFv-CK [9].

How this four amino acid extension is capable of stabilizing the scFv antibody still remains elusive. An interaction with the transmembrane KDEL receptor is unlikely since the binding site of the receptor is exclusively located at the luminal side of the ER membrane [47]. In addition, cytosolic scFv expression could also be improved when the tetrapeptide KDEI was added (scFv₄₈-CI). It was demonstrated before that the substitution of leucine into isoleucine disrupted the tetrapeptide to act as an ER retention signal [13]. It may therefore well be that C-terminal protein degradation is prevented or that the tetrapeptide sterically protects a part of the scFv antibody susceptible for proteolysis.

Although different linker peptides were used to connect the variable domains of the different scFvs this seemed not to be crucial. The scFv₄₈, scFv_{GUS} and the 21C5 scFv-CK [9] and scFv^{Aglyc}-CK all carried almost identical linker peptides. Yet, only the protein expression of the scFv_{GUS} was not improved when the KDEL extension was added. Furthermore, the expression protein level of scFv₅₉-CK was considerably improved when compared to scFv₅₉-C. These scFvs both carried the Gly₂Ser(Gly₄Ser)₃ linker. Susceptibility to proteolytic degradation may therefore depend on the amino acid sequence of the variable heavy and light domains. Since scFv antibodies lack the heavy and light chain constant domains some residues at the former variable and constant domain interface become solvent exposed. Depending on the amino acid sequence in this region proper folding and subsequent overall

stability may be affected, thus increasing susceptibility to proteolytic degradation. Noteworthy in this respect is the recent report in which in COS-1 cells the cytosolic stability of an scFv antibody was greatly improved when the entire C κ domain was added C-terminally [48]. Considering the negative results with scFv₄₉-CK and scFv_{GUS}-CK, apparently not all possible proteolytic sensitive sites are protected by the tetrapeptide extension.

As demonstrated with the cytosolic scFv expression in potato, the positive effect of the KDEL extension on the expression levels does not seem to depend on the choice of the plant species. The processes involved in stabilizing scFv antibodies in the cytosol of different species may be similar and is therefore not a factor determining the success of stable scFv expression.

It can be concluded that the tetrapeptide KDEL or KDEI can have a beneficial effect on the cytosolic expression levels of scFv antibodies in plants. Addition of this four amino acid extension may be a simple and effective solution for cytosolic scFv antibody expression and therefore is worth trying with scFvs which show great promise with respect to their binding affinity but cannot be expressed at a sufficient level in the plant cytosol.

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References

- [1] Tavladoraki, P., Benvenuto, E., Trinca, S., Demartinis, D., Cattaneo, A. and Galeffi, P. (1993) *Nature* 366, 469–472.
- [2] Voss, A., Niersbach, M., Hain, R., Hirsch, H.J., Liao, Y.C., Kreuzaler, F. and Fischer, R. (1995) *Mol. Breeding* 1, 39–50.
- [3] Owen, M., Gandeche, A., Cockburn, B. and Whitelam, G. (1992) *Bio/Technology* 10, 790–794.
- [4] Artsenko, O., Peisker, M., Zurnieden, U., Fiedler, U., Weiler, E.W., Muntz, K. and Conrad, U. (1995) *Plant J.* 8, 745–750.
- [5] Biocca, S. and Cattaneo, A. (1995) *Trends Cell Biol.* 5, 248–252.
- [6] Duan, L.X., Bagasra, O., Laughlin, M.A., Oakes, J.W. and Pomerantz, R.J. (1994) *Proc. Natl. Acad. Sci. USA* 91, 5075–5079.
- [7] Fiedler, U. and Conrad, U. (1995) *Bio/Technology* 13, 1090–1093.
- [8] Bruyns, A.M., Dejaeger, G., Deneve, M., Dewilde, C., Vanmon-tagu, M. and Depicker, A. (1996) *FEBS Lett.* 386, 5–10.
- [9] Schouten, A., Roosien, J., van Engelen, F.A., de Jong, G.A.M., Borst-Vrens, A.W.M., Zilverentant, J.F., Bosch, D., Stiekema, W.J., Gommers, F.J., Schots, A. and Bakker, J. (1996) *Plant Mol. Biol.* 30, 781–793.
- [10] Whitelam, G.C. and Cockburn, W. (1996) *Trends Plant Sci.* 1, 268–272.
- [11] Fecker, L.F., Kaufmann, N., Commandeur, U., Commandeur, J., Koenig, R. and Burgermeister, W. (1997) *Plant Mol. Biol.* 32, 979–986.
- [12] Pelham, H.R.B. (1989) *EMBO J.* 8, 3171–3176.
- [13] Denecke, J., De Ryke, R. and Botterman, J. (1992) *EMBO J.* 11, 2345–2355.
- [14] Wandelt, C.I., Khan, M.R.I., Craig, S., Schroeder, H.E., Spencer, D. and Higgins, T.J.V. (1992) *Plant J.* 2, 181–192.
- [15] Jiang, W.R., Venugopal, K. and Gould, E.A. (1995) *J. Virol.* 69, 1044–1049.
- [16] de Boer, J.M., Smant, G., Goverse, A., Davis, E.L., Overmars, H.A., Pomp, H., Van Gent-Pelzer, M., Zilverentant, J.F., Stokermans, J.P.W.G., Hussey, R.S., Gommers, F.J., Bakker, J. and Schots, A. (1996) *Mol. Plant-Microbe Interact.* 9, 39–46.

- [17] van Engelen, F.A., Molthoff, J.W., Conner, A.J., Nap, J.P., Pereira, A. and Stiekema, W.J. (1995) *Transgenic Res.* 4, 288–290.
- [18] van Engelen, F.A., Schouten, A., Molthoff, J.W., Roosien, J., Salinas, J., Dirkse, W.G., Schots, A., Bakker, J., Gommers, F.J., Jongsma, M.A., Bosch, D. and Stiekema, W.J. (1994) *Plant Mol. Biol.* 26, 1701–1710.
- [19] Florack, D.E.A., Dirkse, W.G., Visser, B., Heidekamp, F. and Stiekema, W.J. (1994) *Plant Mol. Biol.* 24, 83–96.
- [20] Pantoliano, M.W., Bird, R.E., Johson, S., Asel, E.D., Dodd, S.W., Wood, J.F. and Hardman, K.D. (1991) *Biochemistry* 30, 10117–10125.
- [21] Kornfeld, R. and Kornfeld, S. (1985) *Annu. Rev. Biochem.* 54, 631–664.
- [22] Vandenbosch, K.A. (1991) in: *Electron Microscopy of Plant Cells* (Hall, J.L. and Hawes, C., Eds.), pp. 181–218, Academic Press, London.
- [23] Horton, R.M., Cai, Z., Ho, S.N. and Pease, L.R. (1990) *Bio/Techniques* 8, 528–535.
- [24] Rosso, M.N., Schouten, A., Roosien, J., Borst-Vrensens, T., Hussey, R.S., Gommers, F.J., Bakker, J., Schots, A. and Abad, P. (1996) *Biochem. Biophys. Res. Commun.* 220, 255–263.
- [25] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- [26] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) *Proc. Natl. Acad. Sci. USA* 74, 5463–5467.
- [27] Denecke, J., Gosselé, V., Botterman, J. and Cornelissen, M. (1989) *Methods Mol. Cell Biol.* 1, 19–27.
- [28] Iturriaga, G., Jefferson, R.A. and Bevan, M.W. (1989) *Plant Cell* 1, 381–390.
- [29] Laemmli, U.K. (1970) *Nature* 227, 680–685.
- [30] Munro, S. and Pelham, H.R.B. (1986) *Cell* 46, 291–300.
- [31] Lazo, G.R., Stein, P.A. and Ludwig, R.A. (1991) *Bio/Technology* 9, 217–221.
- [32] Flipse, E., Huisman, J.G., de Vries, B.J., Bergervoet, J.E.M., Jacobsen, E. and Visser, R.G.F. (1994) *Theor. Appl. Genet.* 88, 369–375.
- [33] Kuipers, A.G.J., Soppe, W.J.J., Jacobsen, E. and Visser, R.G.F. (1995) *Mol. Gen. Genet.* 246, 745–755.
- [34] Olsder, J. and Hermesen, J.G.T. (1976) *Euphytica* 25, 597–607.
- [35] Rodríguez-García, M.I., Fernández, M.C. and Alché, J.D. (1995) *Planta* 196, 558–563.
- [36] Muesch, A., Hartmann, E., Rohde, K., Rubartelli, A., Sitia, R. and Rapoport, T.A. (1990) *Trends Biol. Sci.* 15, 86–88.
- [37] Rubartelli, A., Cozzolino, F., Talio, M. and Sitia, R. (1990) *EMBO J.* 9, 1503–1510.
- [38] Rubartelli, A., Bajetto, A., Allavena, G., Wollman, E. and Sitia, R. (1992) *J. Biol. Chem.* 267, 24161–24164.
- [39] Czichi, U. and Lennarz, W.J. (1977) *J. Biol. Chem.* 252, 7901–7904.
- [40] Allen, S., Naim, H.Y. and Bulleid, N.J. (1995) *J. Biol. Chem.* 270, 4797–4804.
- [41] Chen, W., Helenius, J., Braakman, I. and Helenius, A. (1995) *Proc. Natl. Acad. Sci. USA* 92, 6229–6233.
- [42] Holst, B., Bruun, A.W., Kielland-Brandt, M.C. and Winther, J.R. (1996) *EMBO J.* 15, 3538–3546.
- [43] Shakin-Eshleman, S.H., Spitalnik, S.L. and Kasturi, L. (1996) *J. Biol. Chem.* 271, 6363–6366.
- [44] Gavel, Y. and Von-Heijne, G. (1990) *Protein Eng.* 3, 433–442.
- [45] Bergman, L.W. and Kuehl, W.M. (1979) *J. Biol. Chem.* 254, 8869–8876.
- [46] Freund, C., Honegger, A., Hunziker, P., Holak, T.A. and Plückthun, A. (1996) *Biochemistry* 35, 8457–8464.
- [47] Singh, P., Tang, B.L., Wong, S.H. and Hong, W. (1993) *Mol. Cell Biol.* 13, 6435–6441.
- [48] Mhashilkar, A.M., Bagley, J., Chen, S.Y., Szilvay, A.M., Helland, D.G. and Marasco, W.A. (1995) *EMBO J.* 14, 1542–1551.