

## Optimization of scFv antibody production in transgenic plants<sup>1</sup>

Ulrike Fiedler<sup>a</sup>, Julian Phillips<sup>b</sup>, Olga Artsaenko<sup>c</sup>, Udo Conrad<sup>d,\*</sup>

<sup>a</sup> Martin-Luther-Universität Halle-Wittenburg, Institut für Biotechnologie, Kurt-Mothes Str. 3, 06120 Halle, Germany

<sup>b</sup> QIAGEN GmbH, Max-Volmer-Str. 4, 40724 Hilden, Germany

<sup>c</sup> University of Maryland Baltimore County, Chemical and Biochemical Engineering, 1000 Hilltop Circle, Baltimore, MD 21250, USA

<sup>d</sup> Institut für Pflanzengenetik und Kulturpflanzenforschung Gatersleben, Arbeitsgruppe Phytoantikörper, Corrensstr. 3, 06466 Gatersleben, Germany

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### Abstract

**Background:** Plants offer various advantages for the production of pharmaceutical proteins over conventional production systems such as bacterial or mammalian cell culture. In order to explore transgenic plants for large-scale production and storage of recombinant antibodies we tried to optimize the accumulation and stability of functionally active single chain Fv (scFv) antibodies in transgenic tobacco plants. **Objectives:** Two different scFv antibodies which were expressed in different plant organs and plant cell compartments have been used for the study. Accumulation levels and antibody properties such as stability and antigen-binding activity were investigated. **Study design:** For ubiquitous expression in tobacco plants, transcription of the scFv genes was controlled by the strong cauliflower mosaic virus (CaMV) 35S-promoter. We used seed specific legumin B4 (LeB4) and the unknown seed protein (USP) promoters from *Vicia faba* for storage organ specific expression. **Results:** High accumulation of the two different scFv proteins in transgenic tobacco plants was only achieved by retention of the recombinant antibodies in the lumen of the endoplasmic reticulum (ER). Expression levels of scFv antibodies reached up to 4–6.8% of total soluble proteins (TSP) in leaves and up to 3–4% in ripe tobacco seeds. Transgenic tobacco seeds as well as tobacco leaves facilitated stable storage of ER-accumulated scFvs over an extended (seeds) or a short (leaves) period of time. Functionally active scFv proteins could be extracted after harvesting of the leaf material—drying and storage for 1 week at room temperature. Both the amount and the binding activity of the scFv proteins remained unchanged. **Conclusion:** A plant expression system where the scFv-proteins are targeted in the ER provides not only the highest accumulation level of active single chain Fv antibodies ever reported but also a short- or long-term storage of the foreign protein in the harvested plant material. © 1997 Elsevier Science B.V.

**Abbreviations:** scFv, single-chain variable fragment; CaMV, cauliflower mosaic virus; LeB4, legumin B4; USP, unknown seed protein; ER, endoplasmic reticulum; KDEL, ER retention signal; lysine-aspartic acid-glutamic acid-leucine; TSP, total soluble protein; C-terminal, carboxy-terminal; ox, 2-phenyloxazol-5-one; ABA, abscisic acid; TIS, translation initiation signal; bp, basepair; EDTA, ethylenediamine tetraacetic acid; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulphate; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; DAP, days after pollination; kDa, kilo Dalton.

\* Corresponding author. Tel.: +49 394 825253; fax: +49 394 825366; e-mail: conradu@ipk-gatersleben.de

<sup>1</sup> The paper is dedicated to Klaus Müntz on the occasion of his 60th birthday.

**Keywords:** Single chain antibody; Plant expression; ER retention; Stability

## 1. Introduction

Single chain Fv antibodies consist of variable light chain and variable heavy chain domains of an antibody molecule fused by a flexible peptide linker [1,2]. These small recombinant antibodies retain full antigen-binding activity but lack specific assembly requirements. They are used in diagnostics and therapeutics [3], as radiolabelled molecules [4] as fusions with immunotoxins [5], enzymes [6–8] or by using scFvs of different specificity to form a so-called bispecific antibody [9]. Today, scFvs can be humanized by replacing the framework region with that of a human antibody [3]. Human scFv antibodies can also be isolated from non-immunized phage display libraries [10].

scFv antibodies have been successfully synthesized in plants and plant cells [11] as well as in bacteria—the more conventional system for recombinant protein production. For pharmaceutical production processes plants offer a number of advantages: no requirement for complex culture media, sterility or large culture vessels (bioreactors), the possibility of composting plant waste material, no contamination with mammalian viruses or bacterial endotoxins. These last two points are especially important for the production of recombinant antibodies for therapeutic use. Expression systems have to be established allowing high-level accumulation of antibodies in transgenic plants. Furthermore, stable short- or long-term storage of antibodies in plant material would be advantageous if the harvested material has to be transported or stored before further processing.

Our general strategy for establishing an optimized plant expression system separately for leaves and seeds was to reduce degradation and improve folding conditions for antibody fragments in leaves and seeds. Because previous results revealed very low or no expression of scFvs in the cytoplasm of plant cells [12–14] we tried to direct the single chain Fv's through the secretory pathway into the extracellular space or retain them in the lumen of the ER.

Two different scFvs have been used in the present study: The scFv-ox which binds to the hapten oxazolone [12] and the scFv-ABA [15] binding to the phytohormone abscisic acid. Previously we have shown that the scFv-ox fused to a signal peptide accumulated up to 0.6% of the total soluble protein in transgenic tobacco seeds [12] and that in transgenic tobacco leaves ER retention caused by the C-terminal retention signal KDEL led to high-level expression of the scFv-ABA [16]. Based on these findings studies for establishing an optimized plant expression system were extended. In this report we present data comparing accumulation levels and stability of these two single chain Fv antibodies in different organs and cell compartments of transgenic tobacco plants. For ubiquitous synthesis, expression of the scFv genes was controlled by the CaMV 35S promoter [17] and by two seed-specific promoters, the LeB4 [18] and the USP promoter [19] of *Vicia faba* for storage organ-specific expression.

The seed-specific promoters differ in strength and temporal activity during seed development. The USP gene from *Vicia faba* is transcribed into the most abundant mRNA species in cotyledons [20]. The USP promoter was shown to be active at early stages of seed development [21] whereas the LeB4 promoter only becomes active during the middle to late stages [12].

The accumulation of the two different scFvs was investigated in leaves and seeds of transgenic tobacco plants, using the three promoters described above, after direction of the recombinant protein into the extracellular space by fusion of the scFv genes with a signal peptide (LeB4 signal peptide) encoding sequence or retention in the ER caused by an additional fusion with the KDEL signal DNA sequence. We show here, that retention in the ER not only leads to high expression in leaves but also results in stable accumulation of the scFv proteins even after simple drying at room temperature. Moreover, favourable high expression and accumulation of scFvs in ripe seeds could be achieved by use of the USP promoter in combination with the ER retention signal KDEL.

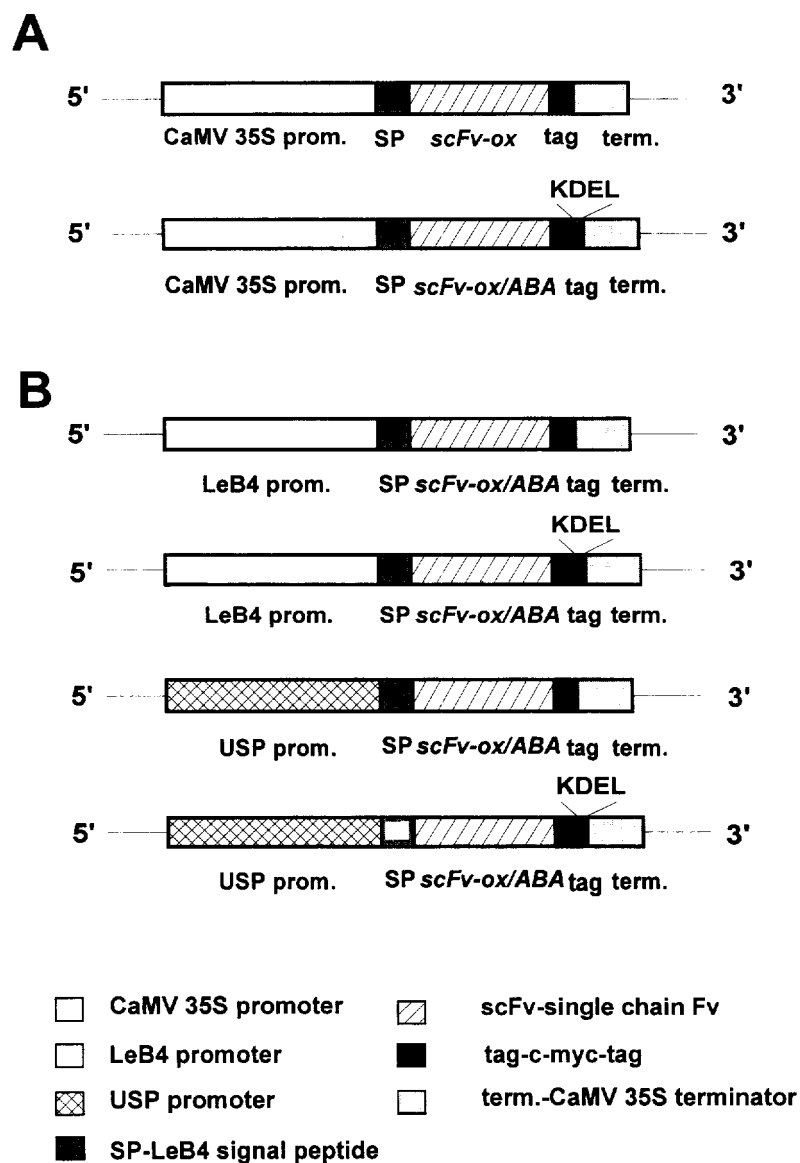


Fig. 1. Schematic representation of designed constructs for ubiquitous (A) and seed-specific (B) expression of the scFv genes in tobacco plants.

## 2. Materials and methods

### 2.1. Construction of plant expression cassettes containing the scFv-ABA and scFv-ox genes

Ubiquitous expression of the scFv genes was controlled by the CaMV 35S promoter (Fig. 1A).

The plasmids pRTRA15 and pRTRA7/3 [16] contain the scFv-ox and scFv-ABA genes, respectively, fused to the LeB4 signal peptide and the KDEL encoding sequences. The plasmid pUF27 containing a signal peptide-scFv-ox gene fusion was constructed as described below. The LeB4 signal peptide encoding sequence was amplified by

polymerase chain reaction (PCR) using the primers 5'-TGACCCGGGAACCATGGCTTCC-AAACCTTTTCTATCTTTGCTTTCA-3' and 5'-GCACCCGGGGGATCCTGCTAAACATGTGCTTGTAAGAGAAGC-3' and pRT103-42-14/S [12] as a template and cloned into the Sma I site of pRT103 [22]. The resulting plasmid was cleaved with Bam HI and ligated in frame with the scFv-ABA encoding fragment [16]. The Nco I/Not I fragment of this plasmid designated pRTLA3/4 was replaced by the Nco I/Not I fragment from pRTRA15.

Seed-specific scFv expression was directed by two different seed-specific promoters, the LeB4-promoter and the USP-promoter (Fig. 1B). The plasmid pRT 103-42-14S/C-scFv-ox harbouring a fusion of the scFv-ox gene and the LeB4 signal peptide encoding sequence under control of the LeB4 promoter was described earlier [12]. Replacement of the Sfi I/Not I fragment of this plasmid by the Sfi I/Not I scFv-ABA fragment from pHEN anti-ABA [15] resulted in pUL9/1. For the ER retention of the scFv-ox and scFv-ABA antibodies the plasmids pRT 103-42-14S/C-scFv-ox and pUL9/1 were cleaved with Xba I and ligated with the DNA fragment encoding KDEL amino acid sequence which was obtained by annealing of the oligonucleotides 5'-CTAGAATCT-GAGAAAGATGAGCTATGAT-3' and 5'-TCA-TAGCTCCTATTTCTCAGACTT-3'. The resulting plasmids were designated pRT103-42-14S/C/K-scFv-ox and pKD5, respectively.

The USP promoter expression cassettes were constructed as follows: an Nco I/Sca I fragment containing 637 bp of the 5'-flanking region and the whole 5'-untranslated region of 51 bp from the USP promoter [19] was ligated to the Nco I/Sca I fragments from pUF27, pRTRA15 and pRTRA7/3, containing the LeB4 signal peptide encoding sequence fused to the scFv-ox gene, the scFv-ox-KDEL and the scFv-ABA-KDEL encoding sequences, respectively. The resulting plasmids were designated pUF11U, pUF20U and pBU10. To construct the signal peptide-scFv-ABA fusion, the Sca I/Stu I fragment from pBU10 containing the USP promoter and 5'-sequence of the scFv-ABA gene was ligated with a Stu I/Sca I fragment

from pUL9/1 containing the 3'-sequence of the scFv-ABA gene, giving rise to pBX6.

In all constructs described the c-myc encoding sequence [23] was fused to the 3'-end of the scFv genes.

## 2.2. Transformation and cultivation of plants

All CaMV 35S and LeB4 promoter expression constructs were cloned as *Hind* III fragments into the *Hind* III site of the binary plant transformation vector pGSGLUC1 [24] and the USP promoter expression constructs into pBin19 [25]. The plasmids were transferred into the *Agrobacterium tumefaciens* strain pGV2260 [26] by electroporation and used for leaf disc transformation [27] of *Nicotiana tabacum* cv. SNN. The integrity of the constructs in *Agrobacterium* was checked by Southern hybridization. Regenerated plants were cultivated under similar conditions in the greenhouse except for plants containing the CaMV 35S promoter-SP-scFv-ABA-KDEL construct. They were grown in the growth chamber under high humidity conditions. Leaf material was harvested from adult plants before flowering and the appearance of yellow leaves. At the same time second generation plants were grown.

## 2.3. Southern analysis

To select transgenic plants harbouring constructs with the seed specific promoters, 20 µg of genomic tobacco DNA was isolated as described by Miller et al. [28] and digested with *Hind* III. To achieve hybridization the cleaved DNA samples were subjected to agarose gel electrophoresis and blotted on to a nylon membrane. Hybridizations were performed with an scFv-ox or scFv-ABA <sup>32</sup>P random labelled probe [29].

## 2.4. Western blot analysis

Total soluble proteins from developing or mature seeds were obtained by grinding seeds with liquid nitrogen and extracting the proteins with seed extraction-buffer (50 mM Tris-HCl, pH 8.0, 200 mM NaCl, 5 mM EDTA and 0.1% Tween 20). Leaf material was homogenized with a drill

and proteins extracted with PBS-buffer pH 7.6. Homogenates were centrifuged at 4°C for 15 min at 14 000 *g* and the concentration of proteins in the supernatants was determined according to Bradford [30]. Equal amounts of protein were separated by SDS polyacrylamide gel electrophoresis, proteins were transferred onto nitrocellulose by electroblotting and the scFv proteins were detected by incubation with the anti-c-myc antibody 9E10 [31], recognizing the c-myc tag fused to the C-terminus of the scFv proteins, and subsequently rabbit anti-mouse antibody peroxidase conjugate according to Fiedler and Conrad [12]. The ECL detection system (Amersham) was used for developing the enzymatic reaction. On the obtained autoradiographs the signal strength of the applied scFv protein extracts and scFv antibodies of known concentration were measured using a pdi white light scanner (Raytest). Only signals corresponding to the molecular weight of scFv monomers (approx. 30 kDa) were taken into account. The expression levels were calculated as % scFv protein of extracted soluble proteins by comparing the intensities of known amounts of scFv antibodies with applied samples using the TINA-Raytest program. As controls, protein extracts from non-transformed tobacco plants were used.

### 2.5. scFv activity analysis

ELISA measurements were carried out to determine the antigen binding activity of plant scFvs. Microtitre plates were coated with the appropriate antigen-BSA conjugate, blocked and incubated with plant extracts containing the expressed scFvs of predetermined concentration. Bound scFvs were detected by anti-c-myc antibody 9E10 and anti-mouse immunoglobulin conjugated to alkaline phosphatase. The enzymatic reaction was performed using *p*-nitrophenylphosphate as substrate and after incubation for 1 h at 37°C the absorbance at 405 nm was measured. The specific antigen binding activities (OD/ $\mu$ g scFv) of scFv proteins extracted from seeds and leaves or differently treated leaves were compared.

### 2.6. Stability analysis

Investigation of scFv protein stability in seeds was related earlier [12]. Ripe seeds were stored at room temperature for around 1 year and protein stability as well as antigen binding activity were determined by Western blot and ELISA measurements.

Leaf material containing the scFv-ABA antibody was harvested from second generation adult plants. Samples were subsequently stored at –20°C, lyophilized or dried for 1 week at room temperature. After drying, proteins were extracted with PBS-buffer pH 7.6 and the scFv-ABA was purified by affinity chromatography according to the protocol described by Artsaenko et al. [16]. From equal amounts of the purified scFv antibodies the ABA-binding activities were determined by ELISA [15]. In a second experiment samples of leaves expressing the scFv-ox antibody were frozen at –20°C or dried at room temperature. Amount and activity of the antibody were determined by Western blot analysis and ELISA as previously described by Fiedler and Conrad [12].

## 3. Results

### 3.1. Chimaeric gene constructs and plant transformation

The constructs for the expression of two scFv genes, scFv-ox and scFv-ABA in transgenic tobacco plants are shown in Fig. 1. The scFvs were cloned into different plant expression vectors for ubiquitous (Fig. 1A) and seed-specific expression (Fig. 1B) in tobacco. The ubiquitous expression being controlled by the strong CaMV 35S promoter and the seed-specific expression either by the LeB4 or by the USP promoter. To investigate the stability and accumulation levels of the antibody proteins in different compartments the scFv genes were fused at their 5'-ends with the LeB4 signal peptide encoding sequence or additionally at the 3'-ends with the KDEL encoding sequence [23] leading to retention of the scFv protein in the ER. The expression cassettes were cloned into the plant transformation vectors pGSGLUC1 [24] or

Table 1

Expression levels (% of total soluble protein) of the scFv-ox and scFv-ABA antibodies in leaves of transgenic tobacco plants

scFv	Highest/average expression levels without KDEL <sup>a</sup>	Highest/average expression levels with KDEL <sup>b</sup>
scFv-ox	0.2/0.1	4.0/1.1
scFv-ABA	Not investigated	6.8/0.75

The ubiquitous expression cassettes contained either scFv gene fusions with the LeB4 signal peptide encoding sequence<sup>a</sup> or an additional ER retention signal encoding sequence<sup>b</sup> under control of the CaMV 35S promoter.

pBin19 [25]. The resulting plasmids were transferred into *Agrobacterium tumefaciens*. After leaf disc transformation, on average 50–100 kanamycin-resistant *Nicotiana tabacum* plants were obtained for each construct, transferred to soil and grown to maturity.

### 3.2. scFv expression analysis in transgenic tobacco leaves

From regenerated CaMV 35S-scFv tobacco plants, soluble leaf proteins were extracted and levels of the scFv protein accumulation were estimated by Western blot analysis. On average 50 expressing plants per construct were used to obtain the mean expression levels shown in Table 1. If the scFv-ox is fused with a signal peptide and the proteins are directed to the extracellular space then scFv protein accumulation levels were relatively low—on average 0.1% of total soluble protein (Table 1). Retention in the ER resulted in a 10–20-fold increase of scFv-ox antibody concentration and also a very high accumulation of scFv-ABA (up to 6.8% of TSP).

The strong seed specificity of the LeB4 and the USP promoter was shown in earlier studies [18,19,32] and confirmed within our study. Expression in leaves is therefore not included in Table 1.

### 3.3. scFv expression analysis in transgenic tobacco seeds

Southern analysis was carried out to identify transgenic tobacco plants. Mature transgenic seeds were harvested, soluble proteins were extracted and scFv protein accumulation was analysed by Western blot. The obtained results

shown in Table 2 include data from an average of 30 expressing plants for each construct. As in leaves, highest scFv levels were measured in mature seeds, when the ER retention signal KDEL was fused to the C-terminus of the scFv protein. If the seed-specific expression was controlled by the LeB4 promoter the average expression levels of both scFvs increased 5–6-fold by using the ER retention signal compared with the situation in seeds of transgenic plants without the KDEL signal at the C-terminus of the scFv protein. These differences in accumulation obtained due to different antibody localization were much more significant when the scFv genes were controlled by the USP promoter. In the USP promoter plants the scFv-ox and scFv-ABA proteins accumulated on average levels of 0.04 and 0.07%, respectively, if the scFv antibodies were directed to pass through the secretory pathway. The average expression levels of antibodies increased 22-fold for scFv-ox and 26-fold for scFv-ABA by retention in the ER. The highest expression levels in ripe tobacco seeds were obtained by using the USP promoter together with the ER retention signal KDEL. Up to 2.6% scFv protein was measured in the case of the scFv-ox antibodies and even higher amounts, up to 4%, in the case of the scFv-ABA.

Accumulation of scFvs was also investigated in mature seeds of transgenic tobacco plants expressing scFv-ABA and scFv-ox in the ER under control of the CaMV 35S promoter (Table 2). The highest expression levels detected in these plants were around 0.75 and 1% of TSP, respectively.

Beside investigations of ripe tobacco seeds, Western blot analysis of proteins from developing tobacco seeds obtained from the second generation were carried out to confirm the different activity of the LeB4 and the USP promoters. As

Table 2

Expression levels (% of total soluble protein) of the scFv-ox and scFv-ABA antibodies in ripe seeds of transgenic tobacco plants

scFv	Highest/average expression levels without KDEL		Highest/average expression levels with KDEL		
	LeB4-P <sup>a</sup>	USP-P <sup>a</sup>	CaMV 35S-P <sup>c</sup>	LeB4-P <sup>b</sup>	USP-P <sup>b</sup>
scFv-ox	0.6/0.2	0.1/0.04	1.0/0.7	1.9/1.0	2.6/0.9
scFv-ABA	0.35/0.04	0.2/0.07	0.75/0.38	0.45/0.19	4.0/1.5

The seed specific expression cassettes contained either scFv gene fusions with a LeB4 signal peptide encoding sequence<sup>a</sup> or an additional ER retention signal encoding sequence<sup>b</sup> under control of the LeB4 promoter (LeB4-P) and the USP promoter (USP-P). For investigation of the scFv accumulation in seeds under control of the CaMV 35S promoter (CaMV 35S-P) only expression cassettes containing scFv gene fusions with both the LeB4 signal peptide encoding sequence and the ER retention signal were used<sup>c</sup>.

shown in Fig. 2 in tobacco seeds from plants harbouring the LeB4 constructs scFv proteins could first be detected around 14–16 days after pollination (DAP). In USP promoter plants, antibody proteins were already detected 10 days after pollination (Fig. 2). Only in the case of the scFv-ABA antibody, high molecular weight bands appeared in seed extracts from DAP 14 until 21 having a size of approx. 80 kDa. We assume that these bands reflect scFv-dimers. Such dimers were not detected in ripe seeds or very young seeds (DAP 7–10). For both scFv-ABA and scFv-ox antibodies expressed in seeds, no degradation products could be detected from Western blot analysis.

### 3.4. Activity and stability analysis

Previous results have demonstrated that scFv proteins extracted from tobacco leaves and seeds bind to the appropriate antigens in ELISA [12,16]. scFv antibodies isolated from mature tobacco seeds had a similar specific antigen binding activity compared with those extracted from leaves. So for example the specific antigen binding activity of scFv-ox antibodies extracted from seeds was determined with 1.8 OD/ $\mu$ g scFv (without KDEL) and 1.4 OD/ $\mu$ g scFv (with KDEL) and for scFvs extracted from leaves with 2.5 OD/ $\mu$ g scFv. Between 34 and 74 transformed plants were included for these determinations.

In this study we focused on the investigation of scFv antibody stability and retention of their specific activities after harvesting of green leaf material. We could demonstrate that retention in the

ER of leaf cells provides excellent protection for the recombinant proteins produced. Therefore we could isolate active scFv proteins from leaf material that was dried at room temperature and stored for over 1 week (Figs. 3 and 4). During the drying process the amount (Fig. 3A) and the specific activity (Fig. 3B) of the scFv-ox proteins remained nearly unchanged. The apparent higher antigen binding activity of the scFv-ox antibodies in dried material (Fig. 3B) can be explained by the slightly higher amount of scFv-ox proteins present in 40  $\mu$ g total soluble proteins from dried leaf material (Fig. 3A). In order to show that scFv-ABA antibodies also retain their activity in dry leaves, their ABA-binding properties were investigated. Total soluble proteins were extracted from leaves dried at room temperature, lyophilized or frozen immediately after harvesting. scFv antibodies were purified from these extracts by affinity chromatography and their antigen binding activities were compared in ELISA (Fig. 4B). The amounts of antibodies equal to those used for activity measurements (approx. 80–100 ng) were compared by Western blot analysis (Fig. 4A). The results clearly show that no loss of antigen binding activity of the scFv-ABA protein can be observed during the storage of leaf material for 1 week at room temperature.

Long-term storage of functionally active antibodies in transgenic seeds bearing the promoter-LeB4 signal peptide encoding sequence-scFv-ox gene construct was reported before [12]. Within our present study we confirmed this finding with an even higher accumulation of scFvs by using the USP promoter and retaining the proteins in the ER.

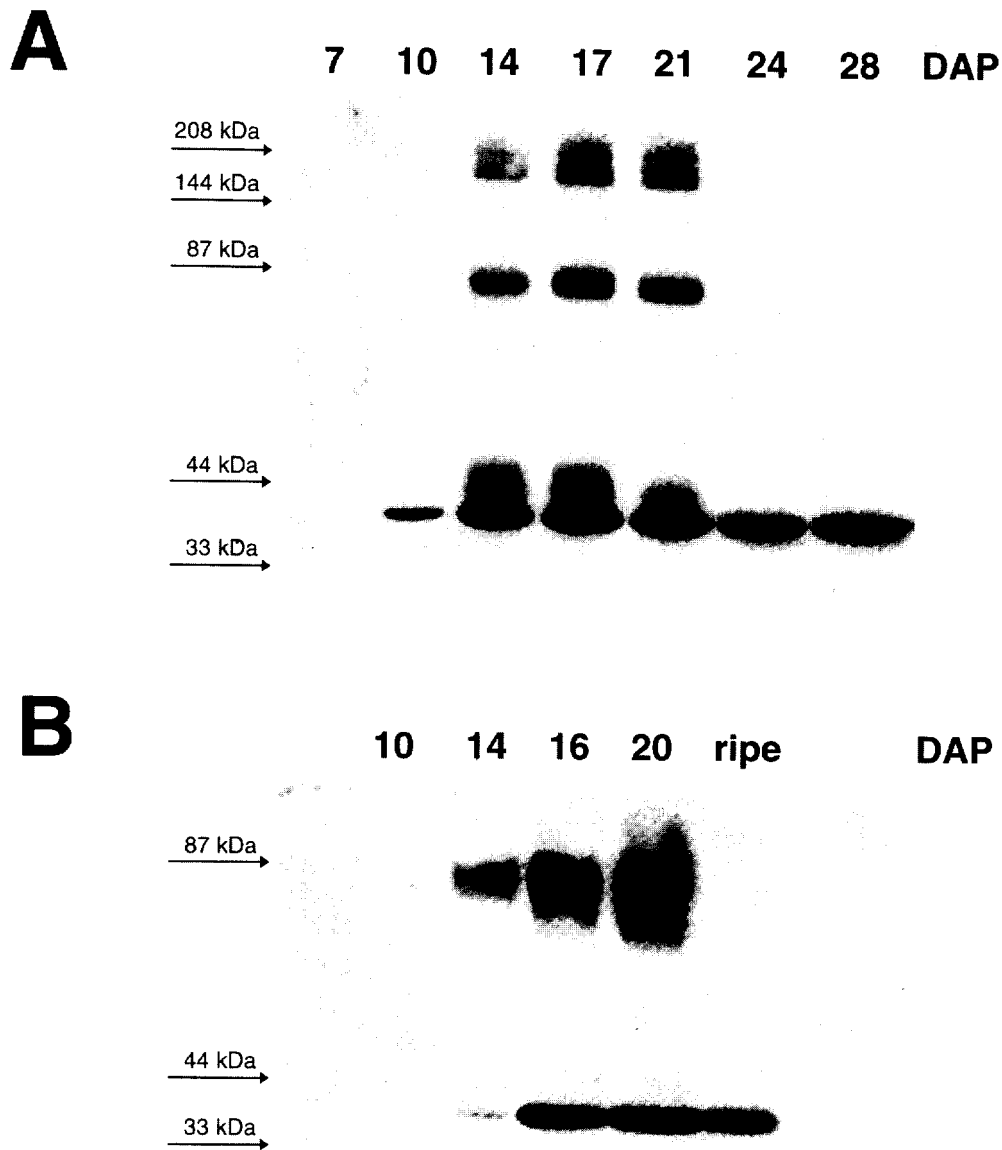


Fig. 2. Accumulation of scFv-ABA proteins during tobacco seed development under control of the USP promoter (A) and the LeB4 promoter (B). (A) Transgenic tobacco seeds obtained from the second generation expressing the LeB4 signal peptide sequence-scFv-ABA gene-ER retention signal sequence fusion under control of the USP promoter were harvested 7, 10, 14, 17, 21, 24 and 28 days after pollination (DAP), proteins were extracted, 10  $\mu$ g TSP separated on an SDS polyacrylamide gel and Western blot analysis was carried out as described in Section 2. (B) Transgenic tobacco seeds obtained from the second generation expressing the LeB4 signal peptide sequence-scFv-ABA gene-ER retention signal sequence fusion under control of the LeB4 promoter were harvested 10, 14, 16 and 20 DAP and after ripening, proteins were extracted and 20  $\mu$ g TSP used for Western blot analysis.



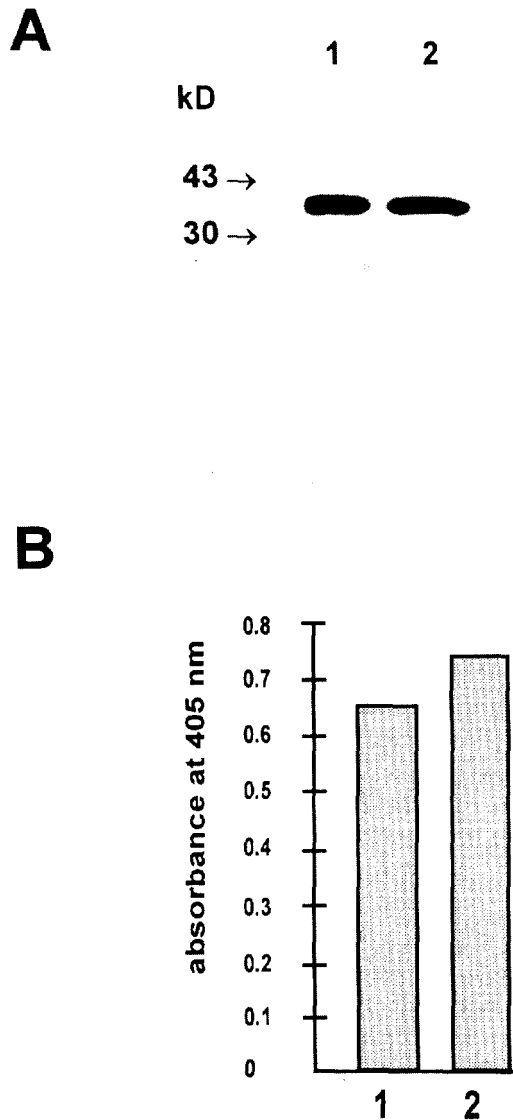


Fig. 3. Stable storage of active scFv-ox protein in dried transgenic tobacco leaves shown by Western blot analysis (A) and ELISA (B). Leaves from transgenic plants of the second generation expressing the signal peptide-scFv-ox-KDEL fusion were harvested, samples were frozen and dried at room temperature. Forty micrograms of extracted protein were used for Western blot analysis (A) and measurement of antigen binding activity by ELISA (B). The average absorbance at 405 nm was obtained by triplicate measurements and subtraction of background absorbance. 1, frozen material; 2, dried material.

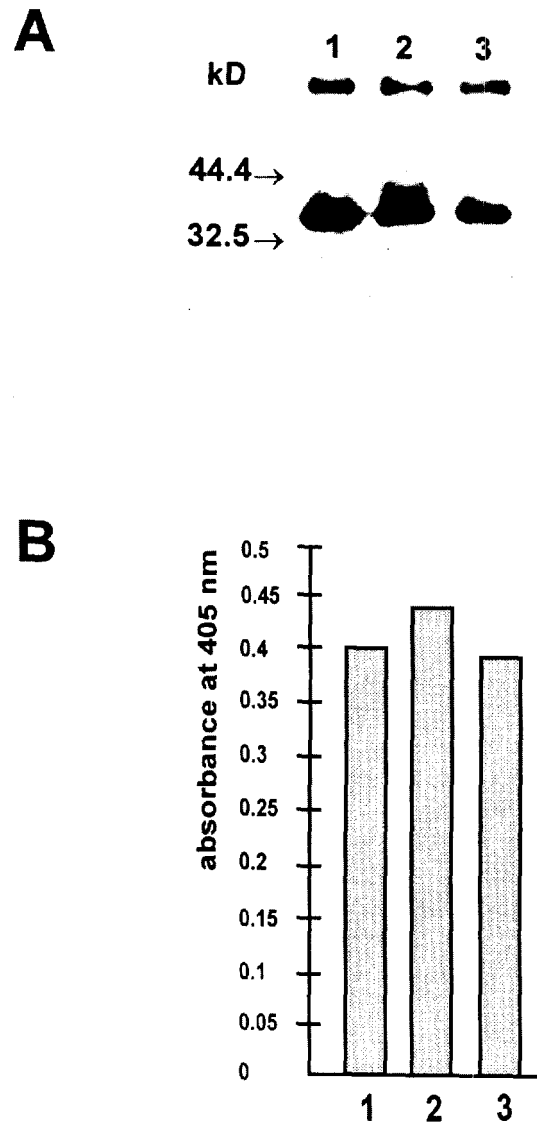


Fig. 4. Antigen binding activity of scFv-ABA antibodies extracted and purified from dried and lyophilized leaves. Leaves from transgenic tobacco plants expressing the signal peptide-scFv-ABA-KDEL fusion were harvested and samples were frozen, lyophilized or dried at room temperature. From the differently treated material protein extracts were made and the scFv-ABA antibodies were affinity purified to remove ABA which would interfere in ELISA. The antigen-binding activity of purified scFv-ABA proteins were tested in ELISA. Amounts of purified scFv antibodies were compared by Western blot analysis (A) and are equal to those used for testing the antigen-binding activity in ELISA (B). Average absorbance at 405 nm was obtained by performing measurements in triplicate and subtraction of background absorbance. 1, frozen material; 2, lyophilized material; 3, dried material.

#### 4. Discussion

The technology of plant genetic engineering offers the pharmaceutical industry exciting new opportunities for producing large quantities of medicinally useful polypeptides with far lower primary energy input than with bacterial, fungal or animal systems. Storage of transgenic plant material and prevention of degradation of transgenic products are important goals of phytoforming technology. Therefore, it is important to synthesize the recombinant proteins in such a way that they can be stably stored for a short or longer time in plants.

We are focusing on the optimization of production and storage of engineered antibodies in plants. In our studies we have chosen the single chain antibodies scFv-ox and scFv-ABA. To analyse the optimal location for high-level accumulation combined with stable storage of the scFv proteins we transformed tobacco plants with constructs containing either LeB4 signal sequence-scFv gene or LeB4 signal sequence-scFv gene-KDEL sequences driven by the CaMV 35S, the LeB4 or the USP promoter. Fusion with signal peptides is sufficient for entry of proteins into the secretory system by translocation across the ER membrane. Further transport depends on specific targeting or retention signals and transport competence [33]. Proteins that lack targeting or retention information are secreted via the bulk-flow or default pathway [34]. This has been shown in transgenic tobacco cells for a number of proteins such as invertase [35], a cytosolic pea seed albumin [36] and scFvs [37].

When we tested protein extracts from leaves for scFv-ox accumulation, both directing to the extracellular space and retention in the ER, resulted in accumulation of the scFv proteins, but with considerably (up to 8–10-fold) higher average expression levels after retention in the ER. The maximal expression level obtained for scFv-ox and scFv-ABA proteins was 4.0 and 6.8% of total soluble leaf protein, respectively. Schouten et al. [38] also obtained a 100-fold increase of scFv accumulation by fusion with the ER retention signal but only to a maximum of 1% of the total soluble leaf protein

in transgenic tobacco. We could confirm the ER localization of the scFv proteins by electron microscopy studies previously only shown for the scFv-ABA protein [16]. Both the scFv-ABA and the scFv-ox proteins were detected in the endoplasmic reticulum and in the nuclear envelope.

Increased expression due to ER retention was also seen with seeds of tobacco plants transformed with LeB4 and USP constructs. With both promoter constructs an increase of scFv protein accumulation in ripe seeds was achieved by retention in the ER: a five-fold increase in the average scFv protein accumulation using the LeB4 promoter and as much as a 20-fold increase with the USP promoter. Highest scFv protein accumulation levels in ripe seeds were measured with constructs containing the USP promoter and an ER retention signal (up to 4% TSP). The presence of the signal peptide probably causes the scFv proteins to enter and to pass through the endomembrane system. We speculate that only a certain proportion of antibody fragments fused to the signal peptide can be correctly folded during their passage through the ER. Incorrectly folded antibody fragments are degraded. Extended retention of scFv proteins in the ER by fusing the KDEL sequence could promote correct folding leading to higher scFv stability and accumulation and less degradation. Another hypothesis is that secretion into the extracellular space does not offer protection from protease activity. In contrast to this Verwoerd et al. [39] showed that the protein phytase fused to a signal peptide accumulates in tobacco leaves up to 14.4% of the total soluble leaf protein and can be isolated from the extracellular fluid 90-fold enriched. However a comparison of expression levels for scFvs and phytase is difficult because expression differences can be influenced by various factors such as transcription and translation efficiencies, folding characteristics and general stability against proteases.

The differences in scFv accumulation between plants transformed with USP and LeB4 promoter constructs may be due to differences in developmental regulation of the promoters. USP promoter plants showed much lower average

expression levels of scFv antibodies in comparison to LeB4 promoter plants if the protein was directed to the extracellular space. Under control of the USP promoter, scFv proteins are synthesized earlier during seed development (Fig. 2A). When proteins are targeted through the secretory pathway without an ER retention signal they are probably secreted into the extracellular space where they do not accumulate to high levels. Possibly, during middle to late tobacco seed development when the LeB4 promoter is active (Fig. 2B) the accompanied loss of water inhibits protein secretion. The majority of scFvs in the seeds of LeB4-scFv transgenic tobacco plants without an ER retention signal are not further secreted but accumulate in the ER and in ER-derived protein bodies. This hypothesis would also be a possible explanation for the results obtained by the electron microscopy studies. Studies with ripe transgenic seeds showed that the scFv-ox protein containing only a signal peptide was localized in the nuclear envelope and in protein accumulating organelles situated near the nucleus, suggesting that these organelles are derived from the ER. If the scFv proteins are retained in the ER from the beginning of synthesis the level of accumulation in seeds increases. This results in higher expression controlled by the USP promoter than in LeB4 promoter plants because of extended time for synthesis and accumulation during seed development. The retention of the transgenic protein in the ER caused by the KDEL signal resulted in the highest accumulation of scFvs in ripe seeds of transgenic plants. Further electron microscopy studies with seeds from KDEL plants are under way and could clarify whether the developmental regulation or the localization of the scFv protein contributes mainly to the high expression levels.

The relatively low activity of the CaMV 35S promoter in seeds could be responsible for the relatively low scFv protein levels determined in seeds of transgenic tobacco plants containing these promoter constructs.

We could show that retention of the recombinant scFv protein in the ER first leads to a considerable increase of accumulation in seeds

and leaves and second causes the extreme stability of the antibodies in dried leaf material.

Transgenic tobacco plants have been shown to tolerate high levels of single chain Fv expression with no apparent detriment to growth or phenotype, when using scFvs which bind to antigens not normally found in plants. In this report we have demonstrated that different scFvs behave similarly concerning their accumulation and stability in different organs. The established expression system offers the possibility of producing large quantities of immunologically active recombinant antibodies in leaves and seeds of transgenic tobacco plants as well as short- or long-term storage of the plant material without any loss of scFv amount or antigen binding activity by retention of the proteins in the ER lumen.

Ongoing studies investigating the number of scFv genes integrated into the plant genome and specific mRNA levels (data not shown) have revealed that different regulatory mechanisms contribute to the scFv protein accumulation in seeds and leaves. Thus, in seeds no correlation was found between the number of integrated transgenes, mRNA levels and recombinant protein accumulation. It suggests that the scFv expression in seeds is not mainly regulated at the level of transcription. In contrast, 35S-promoter plants with high-level scFv expression in leaves contained generally more scFv copies per plant genome and had higher scFv mRNA levels than low-expressing plants. Therefore we assume that further improvement of the expression levels in leaves can be achieved by increasing the amount of specific mRNA using other strong promoters and transcriptional enhancers.

## 5. Conclusions

(1) Retention of the scFv proteins in the ER leads generally to very high expression levels in leaves and seeds of transgenic tobacco plants.

(2) The USP promoter is most suitable for seed specific expression.

(3) Active scFv proteins can be stored in dried leaves and seeds over an extended period of time.

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