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Short communication

Transgenic tobacco plants expressing a dimeric single-chain variable fragment (scFv) antibody against *Salmonella enterica* serotype Paratyphi B

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Abstract

Transgenic tobacco plants were produced that express an anti-*Salmonella enterica* single-chain variable fragment (scFv) antibody that binds to the lipopolysaccharide (LPS) of *S. enterica* Paratyphi B. The coding sequence of this scFv was optimized for expression in tobacco, synthesized and subsequently placed behind three different promoters: an enhanced tobacco constitutive ubiquitous promoter (EntCUP4), and single- and double-enhancer versions of the Cauliflower Mosaic Virus 35S promoter (CaMV 35S). These chimeric genes were introduced into *Nicotiana tabacum* cv. 81V9 by *Agrobacterium*-mediated transformation and 50 primary transgenic (T_0) plants per construct were produced. Among these plants, 23 were selected for the ability to express active scFv as determined by enzyme-linked immunosorbent assay (ELISA) using *S. enterica* LPS as antigen. Expanded bed adsorption-immobilized metal affinity chromatography (EBA-IMAC) was used to purify 41.7 μg of scFv/g from leaf tissue. Gel filtration and surface plasmon resonance (SPR) analyses demonstrated that the purified scFv was active as a dimer or higher-order multimer. In order to identify T_1 plants suitable for development of homozygous lines with heritable scFv expression, kanamycin-resistance segregation analyses were performed to determine the number of T-DNA loci in each T_0 plant, and quantitative ELISA and immunoblot analyses were used to compare expression of active and total anti-*Salmonella* scFv, respectively, in the T_1 generation. As *S. enterica* causes millions of enteric fevers and hundreds of thousands of deaths worldwide each year, large-scale production and purification of this scFv will have potential for uses in diagnosis and detection, as a therapeutic agent, and in applications such as water system purification.

Introduction

The expression of recombinant antibodies (rAbs) and rAb fragments in transgenic plants is recognized for potential cost-effective Ab production and purification for growing market demands (Hiatt et al., 1989; Peeters et al., 2001; Fischer et al., 2004, 2003). Bacteria, yeast, animals and animal cells are major expression systems for these molecules (Hudson, 1998; Houdebine, 2002; Hudson & Souriau, 2003; Krauss 2003; Schillberg et al.,

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2003; Wurm, 2004); however, each system has its own limitation. Bacteria are incapable of producing full-length Abs, which require extensive folding, post-translational modification and multimeric assembly; also, difficulties may arise in separating Abs from bacterial toxins (Schillberg et al., 2002; Schillberg et al., 2003; Baneyx & Mujacic, 2004). Yeast systems lack the ability to efficiently produce properly folded Abs, and have low yields (Peeters et al., 2001; Mayfield et al., 2003; Schillberg et al., 2003), although recent successes are reported with filamentous fungal systems (Gerngross, 2004). Monoclonal Ab (mAb) yields from animals (i.e., milk, eggs) is in the range of 0.4–14 g/L (Peeters et al., 2001); however, the possibility of contaminating an Ab with zoonotic pathogens exists (Fischer et al., 2003). Finally, mammalian cells are the pharmaceutical industry standard for mAb production (Wurm, 2004), but this process can be expensive and slow (Schillberg et al., 2002).

Transgenic plants offer several advantages for Ab production. They can secrete, fold and modify proteins in ways similar to those of mammalian cells (Sheedy & Hall, 2001; Fischer et al., 2004; Gomord & Faye, 2004), and there is reduced potential for zoonotic contamination (Fiedler et al., 1997; Schillberg et al., 2003). Abs produced in plants can also be modified to provide better biological features and produced in large quantities for applications in environmental remediation (Kim et al., 2002; Stranchan et al., 2002; Kramer and Hock, 2003), plant protection (Smolenska et al., 1998; Schillberg et al., 2001; Almquist et al., 2004; Peschen et al., 2004) and medicine.

We chose an anti-*Salmonella enterica* serotype Paratyphi B scFv for production in tobacco plants. A mAb (i.e., Se155–4, Anand et al., 1991a, b; Cygler et al., 1991) was originally developed that binds to the abequose-mannose element of the *Salmonella* serogroup B antigenic determinant (Bundle et al., 1994). A scFv (i.e., B5-1, Deng et al., 1995) was developed from this with two point mutations (i.e., Met34Ile and Gly109Ser) that shifted it from a monomer to a dimer, resulting in high binding affinity for *S. enterica* Paratyphi B. The goals of this research were high-level expression of this scFv, to determine whether transgenic tobacco plants would make the active dimer, and to identify the best candidates for development of homozygous lines for potential large-scale production.

Methods, results and discussion

Transgenic tobacco plants expressing anti-S. enterica serotype Paratyphi B scFv

The B5-1 scFv coding sequence (Deng et al., 1995) was modified for optimal expression in tobacco. The *Arabidopsis* basic chitinase signal sequence (Samac et al., 1990; GenBank Accession AY081519), and QCRL-1 (Hipfner et al., 1996), 6xHis and KDEL (Wandelt et al., 1992) tags were added to the amino and carboxyl termini, respectively. The entire 885 bp sequence (GenBank Accession AY320047), including preferred tobacco codons, was synthesized and subcloned between the EntCUP4 promoter (Foster et al., 1999) and the *nos* terminator in pBIN19 (Bevan, 1984).

Single- and double-enhancer CaMV 35S promoters were PCR-amplified from pFF19 (Timmermans et al., 1990) using primers 35S-*Hin*dIII (5′-AGTGCCAAGCTTGCCAACA-3′) and 35S-*Xba*I (5′-CGCGGTCTAGAAAGCTC-GAGAGAGAGATAGATTTG-3′), and subcloned to replace EntCUP4. Binary plasmids were introduced into *Agrobacterium tumefaciens* strain At542 by electroporation prior to transformation of low-alkaloid *Nicotiana tabacum cv*. 81V9 (Horsch et al., 1986; Miki et al., 1999). Fifty plants for each construct were selected on kanamycin (50 mg/L), and screened for expression of the anti-*S. enterica* scFv by ELISA according to Almquist et al. (2004) using 100 μg/mL *S. enterica* Typhimurium B LPS as specific antigen (Sigma, St. Louis, MO) and Penta.His™ (QIAgen) as primary detection Ab.

Five T_0 plants with the EntCUP4 promoter, eight with the single-enhancer and 10 with the double-enhancer 35S promoter were selected as the highest expressers of active scFv. Specificity of the scFv from these plants was further demonstrated by negative ELISA results using non-specific LPS from *S. enterica* serotype Minnesota (not shown).

Analysis of B5-1 scFv expression

EntCUP4 T_0 plants were produced 4 months earlier than 35S promoter T_0 plants, therefore a comparison of the relative amounts and activities of recombinant scFv required that T_1 seedlings be used. Pooled T_1 sibling plantlets will have an

average number of T-DNA loci approximating that of the original T_0 plant. For instance, T_1 progeny of a single-locus T_0 plant would have a 1:2:1 offspring ratio of homozygous: hemizygous: null-homozygous genotypes at the T-DNA locus, resulting in an average of one T-DNA locus per genome equivalent.

Approximately 200 T_1 seeds from each of the 23 T_0 plants, and from *cv*. 81v9 as a control, were cultured on non-selectable agar medium. After 4 weeks, three replicates of tissue (400 mg each) were harvested from each lawn of T_1 seedlings. The relative protein concentration was determined for all samples, and these were adjusted to the same relative concentration. ELISA was performed to measure relative scFv binding activity per replicate (Figure 1a–c). Total scFv expression was subsequently measured for T_1 seedlings by pooling the three replicate extracts from each line and performing SDS-PAGE followed by quantitative immunoblot analysis, as described by Almquist et al. (2004; Figure 1d–f). Expression and activity of scFv in the T_1 generation of all five EntCUP4 T_0 plants was severely reduced (Figure 1a and d). In contrast, expression and activity were still high in seven of eight single-enhancer plants (numbers 8, 9, 13, 16, 17, 18 and 19; Figure 1b and e), and in five of 10 double-enhancer plants (numbers 24, 28, 30, 31 and 43; Figure 1c and f).

Post-transcriptional gene silencing (PTGS; Neuhuber et al., 1994; Depicker & Van Montagu, 1997; Jones et al., 1999) is a likely explanation for the reduction of transgene expression in double-enhancer 35S plants in the T_1 generation, as this phenomenon is typical of a strong, constitutive promoter (Elmayan & Vaucheret, 1996). For e.g., double-enhancer plant 34 showed no active or total scFv in the T_1 generation (Figure 1c and e); this plant line should be especially susceptible to PTGS as quantitative real-time PCR analysis (not shown) estimated 25 T-DNA copies in the T_0 progenitor. We have not investigated the reason for decreased expression levels among the EntCUP4 plants, but suggest transcriptional gene silencing (TGS) as a possible cause because tCUP sequences are similar to RENT sequences (repetitive elements from *Nicotiana tabacum*; 93% identity over 365 nucleotides; data not shown). If T-DNA insertions occured near methylated RENT sequences in the tobacco genome, tCUP sequences may therefore become methylated, leading to TGS (Ingelbrecht et al., 1999). Although it would be reasonable to suggest that the EntCUP4 plantlets produced very little scFv due to their age (4 weeks) or being raised on agar medium, we suggest these possibilities are unlikely because one EntCup4 T_1 family was grown to maturity in a greenhouse, and ELISAs performed on over 20 individuals showed they all had no active scFv (unpublished).

Nickel affinity purification and quantification of B5-1 scFv

Protein extractions from 100 g of leaves from each of three T_0 plants, i.e., single-enhancer plants 7, 18 and 19, were pooled and subjected to EBA-IMAC (Jiang & Hearn, 1996; Valdes et al., 2003; Sahin et al., 2005). ScFv was eluted in a buffer containing 300 mM imidazole and fractions of 6 mL were collected and assayed for activity. Ten ELISA-positive fractions were pooled and analyzed by SDS-PAGE in comparison with the crude starting material (Figure 2a). The concentration of purified scFv was estimated using quantitative immunoblot technology, in comparison with known amounts of 6xHis Protein Ladder standard (QIAGEN). A single 30 kDa band was detected with the Penta.-His™ Ab (Figure 2b, arrow), whereas wild-type control extract did not contain an immunopositive band (not shown). Densitometric analysis indicated 41.7 μg of purified scFv/g of mature leaf biomass. Identification of the purified scFv was further verified by MALDI-TOF mass spectrometry, since 114 of the 287 amino acids in the scFv sequence were inferred from the masses of five peptide fragments (not shown; Bauer & Kuster, 2003).

Active B5-1 scFv is a dimer or higher-order multimer

Preparations of the anti-*Salmonella* scFv expressed in tobacco and *E. coli* (not shown) were separated by Superdex 75 (Amersham Biosciences, Piscataway, NJ) gel filtration chromatography. Note that scFv expressed in *E. coli*, as described by Deng et al., (1995), did not contain the C-terminal KDEL, 6xHis and QCRL-1 tags. UV traces were read at 280 nm and peaks containing expected monomers, dimers and higher-order multimers

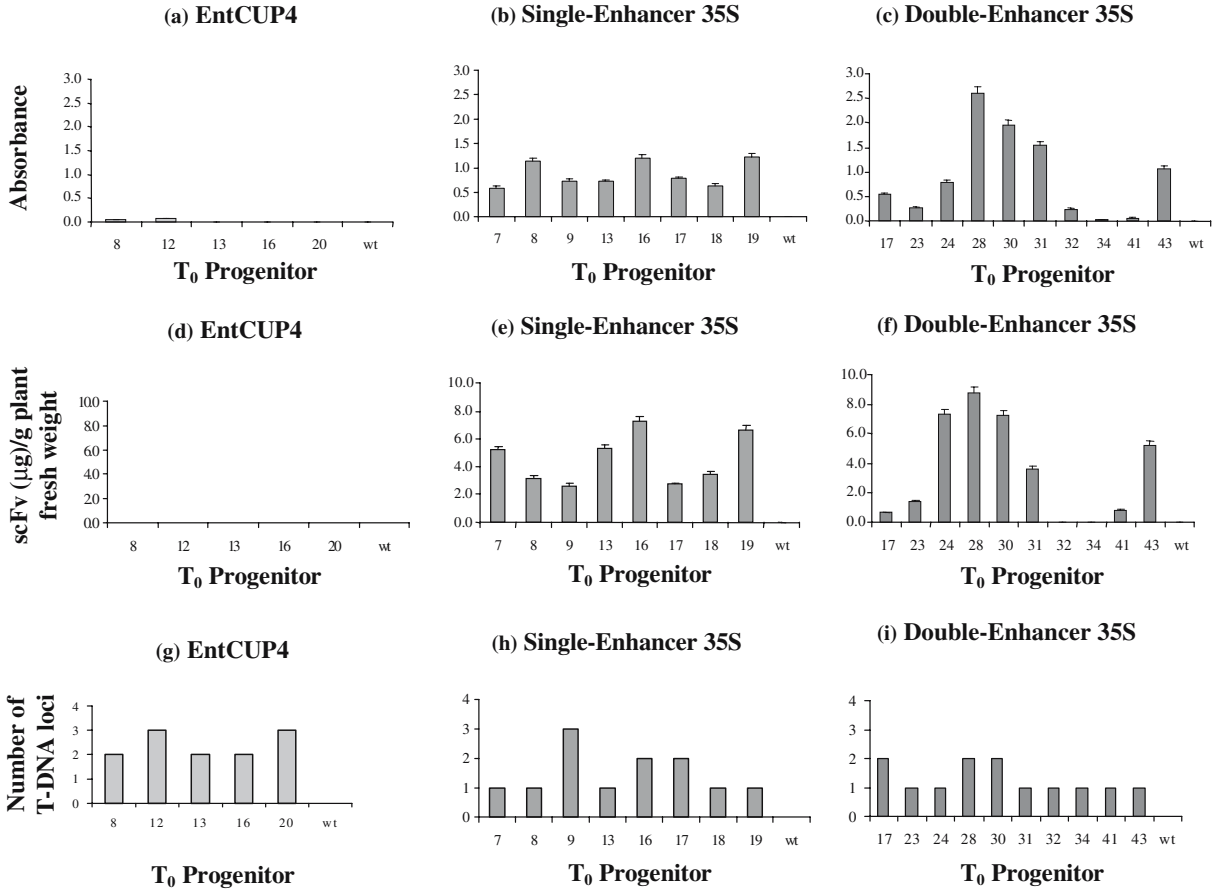

Figure 1. Comparison of active and total anti-*Salmonella enterica* Paratyphi B scFv in the T_1 generation, and number of T-DNA loci in T_0 progenitor plants. (a–c) ELISA absorbance values indicating scFv activity, with standard errors, for protein preparations from T_1 sibling sets of five EntCUP4 (a), eight single-enhancer 35S (b) and 10 double-enhancer 35S (c) plants. Relative absorbances are given at the left, and T_0 progenitors of T_1 sibling sets are given at the bottom. (d–f) Total scFv in µg/g plant fresh weight with standard errors, indicated at the left, for T_1 sibling sets, as determined with quantitative immunoblotting technology. (g–i) Number of T-DNA loci, indicated at the left, for T_0 progenitor plants, as determined with kanamycin-resistance segregation assays (wt: wild type *N. tabacum cv.* 81v3).

were identified by comparative elution with a protein size standard (Figure 2c). SPR analyses were performed with a BIACORE 3000 biosensor system (Biacore, Inc., Piscataway, NJ) on fractions containing these peaks to determine whether they contained active scFv (Figure 2d). BSA-*S. enterica* Essen O-chain (serogroup B) was immobilized at a surface density of 9000 response units (RUs) on research grade CM5 sensor chips (Biacore) at a concentration of 100 µg/mL in 10 mM acetate, pH 4.5, by amine coupling according to the manufacturer's instructions. The reference surface had 10,000 RUs of BSA immobilized under the above conditions. Analyses were carried out at 25°C in 10 mM HEPES (pH 7.4) containing 150 mM NaCl and 3 mM EDTA at a flow rate of 20 µL/min. Chip surfaces were regenerated with 100 mM HCl for 3 s.

Gel filtration and SPR analyses showed that active B5-1 scFv from *E. coli* eluted as a dimer or higher-order multimers in one major peak, which was divided into four fractions (EF1 through E4; Figure 2c and d). Several plant fractions were collected that had scFv activity: PF2, co-eluted with higher-order multimers; PF3, with the dimer; PF4, with the monomer (Figure 2d). SPR showed that scFv from PF2, PF3 and EF3 had high functional affinity for the *S. enterica* Essen B O-chain, as indicated by their slow dissociation phases (Figure 2d). Expanded response traces on the

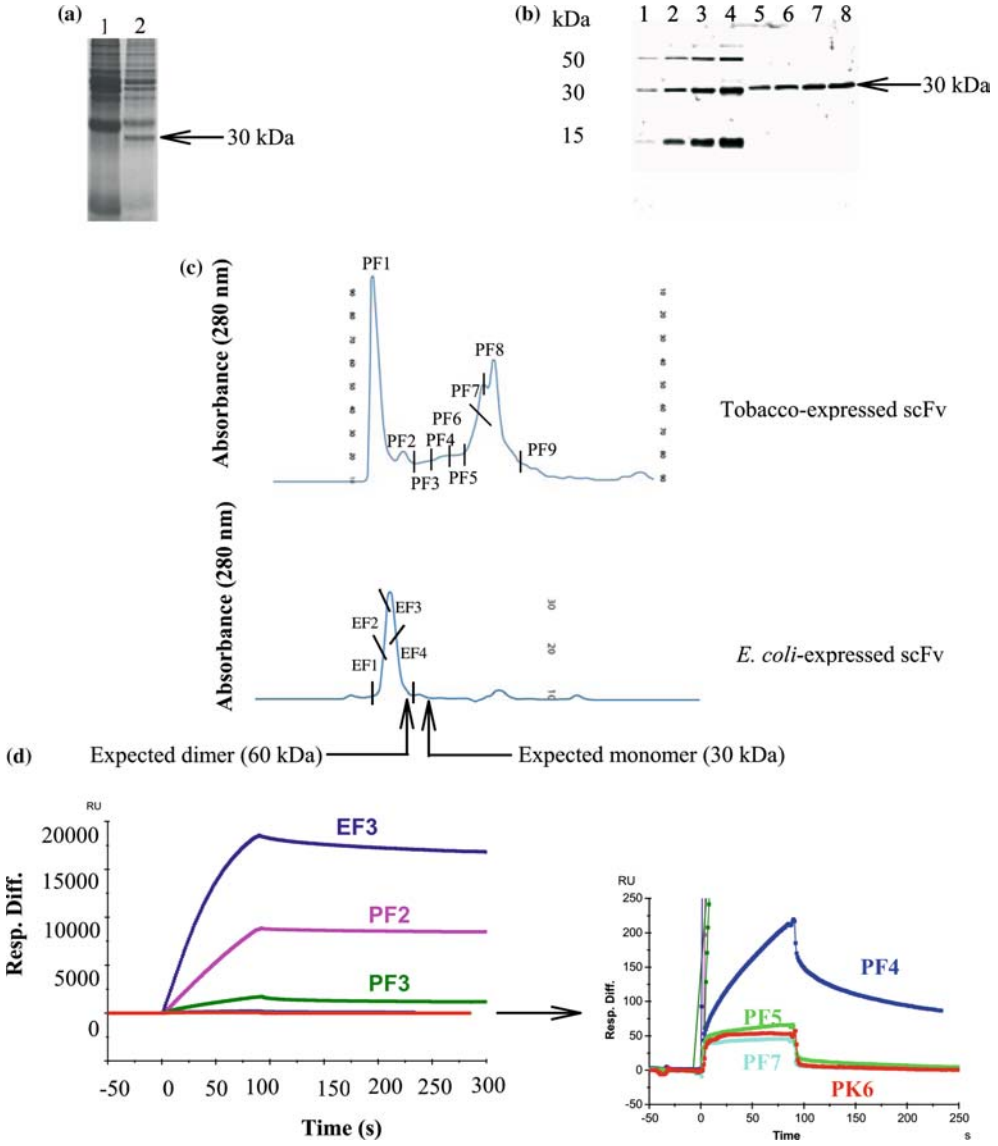

Figure 2. Purification, quantification, gel filtration and surface plasmon resonance (SPR) analyses of anti-*Salmonella enterica* Paratyphi B scFv. (a) Coomassie blue-stained denaturing SDS-PAGE. Lane 1, crude extract; lane 2, pooled sample of immunopositive fractions from expanded bed absorption-immobilized metal affinity chromatography (EBA-IMAC) purification. The 30 kDa scFv is indicated by the arrow on the right. (b) Quantitative immunoblot analysis. Lanes 1–4, 6xHis Protein Ladder (QIAgen), loaded with quantities of 100, 200, 300 and 400 ng, respectively; lanes 5–8, pooled EBA-IMAC fractions, loaded with 2.5, 5.0, 7.5 and 10 μL, respectively. Immunopositive bands were detected using Penta.His (QIAgen) as primary antibody; arrow at right indicates 30 kDa scFv. Migration of molecular weight size standards is shown on the left. (c) Gel filtration of anti-*S. enterica* scFv expressed in tobacco and in *E. coli*. Absorbances at 280 nm are shown at the left. Plant fractions (PF) are indicated by PF1 through PF9, and *E. coli* fractions (EF) as EF1 through EF4. Arrows on the bottom indicate where the monomeric and dimeric scFvs were expected to elute. (d) SPR analyses of EF3 and PF2 through PF7. Selected fractions eluted in (c) were passed over BSA-*S. enterica* Essen O-chain (serogroup B) bound to the surface of a CM5 sensor chip. Expanded sensorgram for PF4 through PF7 is shown at the right of (d). Response differences are at the left and time in seconds is at the bottom of each sensorgram.

right of Figure 2d indicate that PF4 displayed weaker functional affinity for bound antigen, and PF5 through PF7 had no affinity, as they had rapid dissociation phases. These results are consistent with published data on this scFv, which show the dimeric form has higher binding affinity ($K_D = 8 \times 10^{-8}\ M^{-1}$) than the monomer ($K_D = 4 \times 10^{-6}\ M^{-1}$; Deng et al., 1995), presumably

due to increased avidity. Our results indicated that the active scFv expressed by tobacco was predominantly a dimer or higher-order multimer.

Some of the T_1 lines expressed scFv with less functional activity than others, even though they expressed similar amounts of total scFv. For e.g., double-enhancer lines 24 and 30 had similar total scFv (Figure 1f), yet line 24 expressed only half the active scFv of line 30 (Figure 1c). Single-enhancer lines 7, 9 and 13 showed a similar trend; i.e., all three lines showed similar activity (Figure 1b), while line 9 expressed only half the total scFv of the other two (Figure 1e). It is possible that some of the T_1 lines expressed proportionately more monomeric than dimeric or higher-order anti-*Salmonella* scFv, and thus their apparent reduction in functional scFv activity was due to differences in overall avidity (Arndt et al., 1998). We have not investigated this phenomenon further.

Identification of T_1 seed suitable for development into lines expressing B5-1 scFv

T_0 tobacco plants were characterized to determine T_1 seed that would be suitable for development into lines that express functional scFv. The number of T-DNA loci in each T_0 plant was estimated by kanamycin-resistance segregation analysis (not shown), where approximately 200 T_1 seeds from each T_0 plant were cultured on MS agar medium containing kanamycin (100 mg/mL). After 5 weeks, ratios of resistant to susceptible seedlings were determined and the numbers of T-DNA loci were calculated, based on Mendelian inheritance models for one to four independently segregating loci. This method showed 12 of the scFv-expressing T_0 plants contained only one genetic locus with an active *npt*II gene, while eight had two loci and three had three loci (Figure 1g).

For the development of such lines, transgenic plants should have heritable, high expression of functional scFv and contain a small number of T-DNA copies or loci. After self-pollination, lines would be developed from homozygous T_1 plants. Single-locus homozygotes improve the chances of maintaining high scFv expression levels since the possibility of gene silencing is reduced (Meyer & Saedler, 1996); multilocus T-DNA plants can over-express the T-DNA-encoded mRNA, resulting in PTGS (Flavell, 1994). In addition, T_0 plants with single T-DNA insertions are much easier to develop to homozygosity. Although 10 of 12 single-locus T_1 families expressed measurable active and total scFv, those from single-enhancer T_0 plants 8 and 19, and from double-enhancer plants 31 and 43 are the best candidates for development of homozygous lines (Figure 1).

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