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Transient plant production of *Salmonella* Typhimurium diagnostic antibodies

Lilya Kopertekh[a,*], Torsten Meyer[b,1], Cornelia Freyer[a], Michael Hust[b]

[a] *Julius Kühn-Institut, Bundesforschungsinstitut für Kulturpflanzen, Institut für die Sicherheit biotechnologischer Verfahren bei Pflanzen, Erwin-Baur-Str. 27, 06484, Quedlinburg, Germany*

[b] *Technische Universität Braunschweig, Institut für Biochemie, Biotechnologie und Bioinformatik, Abteilung Biotechnologie, Spielmannstr. 7, 38106, Braunschweig, Germany*

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ABSTRACT

Salmonella Typhimurium is one of the most important zoonotic pathogens worldwide and a major cause of economic losses in the pig production chain. The emergence of multi-drug resistant strains over the past years has led to considerations about an enhanced surveillance of bacterial food contamination. Currently, ELISA is the method of choice for high throughput identification of *S.* Typhimurium. The sensitivity and specificity of this assay might be improved by application of new diagnostic antibodies. We focused on plant-based expression of candidate diagnostic TM43-E10 antibodies discovered using as antigen the *S.* Typhimurium OmpD protein. The scFv-TM43-E10 and scFv-Fc-TM43-E10 antibody derivatives have been successfully produced in N. benthamiana using a deconstructed movement-deficient PVX vector supplemented with the γb silencing suppressor from Poa semilatent virus. The plant-made antibodies showed the same antigen-binding specificity as that of the microbial/mammalian cell-produced counterparts and could recognize the OmpD antigen in *S.* Typhimurium infected plant samples.

1. Introduction

Salmonella enterica, subspecies *enterica* serotype Typhimurium can infect both animals and humans and cause food-borne gastrointestinal infections, usually through poultry, beef, pork, milk and eggs. It can also be found in non-alcoholic beer or seafood. Human infections with phage type *S.* Typhimurium DT104 are particularly critical, because this strain is resistant to most of the commonly used antibiotics [1]. Therefore, continuous monitoring of bacterial food contamination is necessary to prevent infections in humans. Established methods for *S.* Typhimurium diagnostics are time-consuming and use microbiological cultures on different liquid and solid media [2], specific fluorescence labeled DNA probes [3] or PCR [4]. Currently, high throughput diagnostics of *S.* Typhimurium is performed by indirect ELISA [5]. The commercially available ELISA kits, SALMOTYPE®- or Enterisol®-ELISA, use a mixture of O-antigens of *Salmonella enterica* subspecies *enterica* serovars. Because of this mixture, cross-reactions occur with other bacteria [6]. In addition, the sensitivity varies between the different ELISA assays [7].

For a sensitive and specific ELISA, new immunogenic and species-specific proteins are required. One of the major proteins of the *S.* Typhimurium outer membrane, the 39 kDa OmpD protein, is a promising candidates to develop corresponding diagnostic antibodies. It is expressed in addition to OmpF and OmpC proteins and is shown to be immunogenic [8,9]. Recently, human recombinant antibody fragments (scFv) were isolated from the naive human antibody gene library HAL7/8 by phage display using the OmpD protein as an antigen [10]. The scFv-TM43-E10 antibody was further characterized with the aim to develop diagnostic assay [11].

Several expression systems have been developed so far to produce recombinant antibodies including bacterial, yeast, insect and mammalian cell cultures [12]. Over the past two decades plants have emerged as an alternative production platform. The major advantages of plants over traditional expression systems are low production costs, flexible scalability and eukaryotic type of posttranscriptional modification [13,14].

Recombinant proteins in plants can be produced using two main expression methods: stable transformation and transient expression [15–17]. Amongst the several expression approaches in plants transient expression techniques, especially techniques based on plant virus vectors, made the most significant progress in recent years. Two

* Corresponding author.
E-mail address: lilya.kopertekh@julius-kuehn.de (L. Kopertekh).

[1] Present address: Testo industrial services GmbH, Gewerbestraße 3, 79199, Kirchzarten, Germany.

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main approaches, "full-length" strategy and deconstructed strategy, have been used to design virus vectors [18–20]. Deconstructed versions of RNA viruses like, *Potato virus* X [21], *Tobacco mosaic virus* [22,23], *Cowpea mosaic virus* [24], as well as DNA viruses like *Beet curly top virus* [25], have been developed and successfully applied to produce recombinant proteins in plants. In the most advanced version the "deconstructed virus vectors" are combined with *A. tumefaciens*-mediated delivery into plant cells [26].

The main goal of this study was to evaluate a feasibility of transient expression system based on the movement-deficient PVX vector supplemented with the γb silencing suppressor for the production of the TM43-E10 recombinant antibody. Two TM43-E10 antibody formats, namely single chain variable fragment (scFv) and scFv fused to the lgG Fc moiety (scFv-Fc), were designed, expressed in *N. benthamiana* and functionally characterized. The scFv is a smallest of the recombinant antibody formats, which is capable of antigen binding. It consists only of the variable (V) antibody regions (VL and VH) connected with a short linker peptide. The scFv-Fc fragment combines the VL, VH and Fc regions of the IgG. The scFv-Fc format might offer several advantages over the phage display-derived scFv, including bivalent binding, longer half-life and Fc-mediated effector functions [12]. Smaller antibody fragments have several advantages such as possible application of different antibody generation systems for selection/design, easier production and full antigen binding capacity of IgG [12,27–29].

2. Materials and methods

2.1. Construction of modified PVX vectors

The pLH-PVX-m vector was constructed by overlap PCR using PVX-AvrI-forw/PVX-ovl-rev and PVX-ovl-forw/PVX-SacI-rev primer pairs (Table S1) and a pPVX-201 plasmid [30] as a template. The amplification products were mixed and subjected to a second PCR with PVX-AvrI-forw/PVX-SacI-rev primers. The final PCR fragment was inserted into the pUC-AP [31] vector yielding the pUC-3′-PVX-m plasmid. The *Avr*II-*Sac*I fragment of pUC-3′-PVX-m was ligated into pPVX-201 resulting in pPVX-201-m. The modified PVX sequence was transferred into the binary vector pLH-Δbar [32] by ligation of a T4 DNA polymerase treated *Sph*I-*Ehe*I fragment of pPVX-201-m and *Stu*I digested pLH-Δbar plasmid. The resulting vector was designated as pLH-PVX-m.

In order to design the expression vector containing a PVX modified sequence and the γb gene silencing suppressor from *Poa semilatent virus* (PSLV) a γb amplicon was generated using SalI-BγP-forw and SpeI-BγP-rev primers and pPγb plasmid [33] as a template. The PCR product was cut with *Sal*I and *Spe*I restriction enzymes and cloned into the *Xho*I-*Xba*I digested pRT103 plasmid generating the pRT-35S-γb-ter construct [34]. A 35S-γb-ter expression cassette was released from the pRT-35S-γb-ter plasmid by *Pst*I and inserted into *Nsi*I digested pLH-Δbar binary vector resulting in pLH-γb plasmid. The PVX-m sequence from pPVX-201-m was digested with *Sph*I-*Ehe*I, treated with T4 DNA polymerase and cloned into the *Stu*I restricted pLH-γb plasmid yielding the pLH-γb-PVX-m transient expression vector.

2.2. Expression vectors

To design plant expression vectors the scFv-TM43-E10 and scFv-Fc-TM43-E10 antibody fragments were amplified using SalI-scFv-forw/EcoRV-scFv-rev and XhoI-scFv-Fc-forw/EcoRV-scFv-Fc-rev primers, respectively (Table S1). The template plasmids were as following: pOPE101-TM43-E10, pCMV2.5-hIgG1Fc-scFv-Fc-TM43-E10 for scFv-TM43-E10 and scFv-Fc-TM43-E10 antibody fragments, respectively. Details of designing pOPE101-TM43-E10 and pCMV2.5-hIgG1Fc-scFv-Fc-TM43-E10 plasmids can be found in the Supplementary Materials. The *Sal*I-*Eco*RV digested scFv-TM43-E10 PCR product were incorporated into the *Sal*I-*Eco*RV restricted pBluescript II SK(-) plasmid. Cloning the pSK-scFv-Fc-TM43-E10 plasmid was performed by ligation of a scFv-Fc-TM43-E10 PCR fragment and pBluescript II SK(-) plasmid both restricted with *Xho*I-*Eco*RV. Antibody fragments were released from the pBluescript II SK(-) vector by *Xho*I-*Xba*I (scFv-TM43-E10) and *Sal*I-*Spe*I (scFv-Fc-TM43-E10) and ligated into *Xho*I-*Spe*I treated pLH-PVX-m and pLH-γb-PVX-m plasmids. Four final expression vectors were designated as pLH-PVX-m-scFv-TM43-E10, pLH-PVX-m-scFv-Fc-TM43-E10, pLH-γb-PVX-m-scFv-TM43-E10, and pLH-γb-PVX-m-scFv-Fc-TM43-E10.

2.3. Agroinfiltration procedure

A. tumefaciens (strain LBA4404) cultures carrying plant expression vectors were grown overnight in LB medium supplemented with appropriate antibiotics, collected by centrifugation, resuspended in agroinfiltration buffer (10 mM MES, pH 5.7, 10 mM $MgCl_2$ and 150 μM acetosyringone) to an OD_{600} of 0.5 and incubated for 2 h at room temperature. Middle leaves of six-week old *N. benthamiana* plants were infiltrated using a syringe without a needle.

2.4. Western blot analysis

Leaf samples were homogenised in 250 mM Tris HCl (pH 7.8), mixed with equal volume of Laemmli buffer (2x), boiled for 5 min, clarified by centrifugation and separated on a 12% SDS polyacrylamide gel. Each sample was comprised of three discs obtained from the different agroinfiltrated leaves of one plant. Coomassie staining was performed by Coomassie blue. The scFv-TM43-E10 blots were probed with an anti-c-Myc mouse antibody (Sigma-Aldrich). The scFv-Fc-TM43-E10 membranes were developed using an anti-human lgG (Fc) mouse antibody (Sigma-Aldrich). Cross-reacting protein bands with secondary anti-mouse goat antibody (Sigma-Aldrich) were visualized with BCIP/NBT color developing reagent.

2.5. Purification of recombinant proteins

The OmpD protein and scFv-TM43-E10 antibody format were produced in *E. coli* in shaking flasks according to Meyer et al. [7] and purified by IMAC. The scFv-Fc-TM43-E10 fragment was produced in HEK293–6E cells as described previously [35] and isolated by protein A affinity purification.

Agroinfiltrated leaf material was harvested on 6 dpi, homogenized and extracted in extraction buffer (20 mM phosphate buffer, 150 mM NaCl, pH 7.2). Leaf extracts were clarified by centrifugation at 6000 *g* for 20 min at 4 °C and filtration through 0.2 μm filter. Clarified leaf extracts were subjected to IMAC (scFv-TM43-E10) and Protein A (scFv-Fc-TM43-E10) purification. The scFv-TM43-E10 antibodies were eluted with extraction buffer containing 250 mM imidazol. The scFv-Fc-TM43-E10 protein was eluted with 100 mM sodium citrate buffer, pH 3.0. Purified scFv-TM43-E10 and scFv-Fc-TM43-E10 antibody fragments were further analysed by size-exclusion chromatography (SEC) on Superdex 200-16/60 column (GE Healthcare). Calibration was done with Cytochrom C (12.4 kDa), Carbonic Anhydrase (29 kDa), Albumin (66 kDa) and Alcohol Dehydrogenase (150 kDa). The eluted fractions after SEC were investigated by reduced SDS-PAGE and Coomassie blue staining.

2.6. ELISA

Antigen binding of TM43-E10 antibody formats was determined by ELISA in 96-well microtitre plates (Greiner Bio-One)

coated overnight at 4 °C with OmpD recombinant protein or plant material infected with S. Typhimurium. After coating the wells were washed three times with phosphate buffer (PBST) and blocked through 2 h incubation in PBST supplemented with 2% skimmed milk (PBST-M). Blocked microtitre plates were washed three times with PBST-M and incubated with purified antibody fragments for 2 h at room temperature. After further washing bound scFv and scFv-Fc were detected with the anti-c-Myc mouse and anti-human lgG (Fc) mouse antibody, respectively, followed by the addition of anti-mouse goat antibody. The visualization was carried out with p-nitrophenyl phosphate as substrate and the reaction was stopped by adding 2 M NaOH. Absorbance at 450 nm was measured by using a SUNRISE ™ microtitre plate reader (Tecan).

For antigen titration ELISA different dilutions of OmpD recombinant protein ranging from 0.1 ng/ml to 1000 ng/ml were coated in duplicates to wells overnight at 4 °C. The detection was carried out with a concentration of antibody at half maximum saturation (determined by antibody titration ELISA) as described above. 56 nM of scFv-TM43-E10 and 25 nM of scFv-Fc-TM43-E10 antibody were used in the experiments. The antibody detection limit was defined as 3 SDs above the background. Antigen and antibody titration ELISA tests have been repeated twice.

Quantitative ELISA protocol to determine the accumulation of scFv-TM43-E10 and scFv-Fc-TM43-E10 antibody fragments in leaf tissue is provided as Supplementary Material.

2.7. qPCR analysis

RNA for qPCR analysis was isolated from agroinfiltrated *N. benthamiana* leaves taken at 5 dpi using the RNeasy Mini Kit from Qiagen (Qiagen) according to manufacturer's specifications. The first strand cDNA was synthesized using Maxima Reverse Transcriptase and random hexamer primer with 2 μg of total RNA in a reaction final volume of 20 μl following manufacturer instructions (Thermo Scientific). Real-time PCR reactions were performed with Mastercycler® ep realplex (Eppendorf). Reaction mixture contained 2 μl of cDNA, 10 μl of 2x Maxima SYBR Green qPCR Master Mix (Thermo Scientific) and 0.8 μl of 10 μM primers in a total volume of 20 μl. Primer sequences for the *TM43-E10* and reference cyclophilin (*cyp*) genes are listed in Table S1. PCR thermal cycles were as follows: initial denaturation step for 10 min at 95 °C followed by 45 cycles of denaturation for 15 s at 95 °C, annealing for 30 s at 60 °C and extention for 30 s at 72 °C. Relative quantifications were performed based on the ΔΔCT method [36] using *cyp* gene as an internal standard. Three biological replicates and three technical replicates for each sample were analysed by qPCR.

3. Results

3.1. Expression of TM43-E10 antibody fragments in plants

The scFv-TM43-E10 and scFv-Fc-TM43-E10 coding sequences were cloned into pLH-PVX-m and pLH-γb-PVX-m plasmids (Fig. 1).

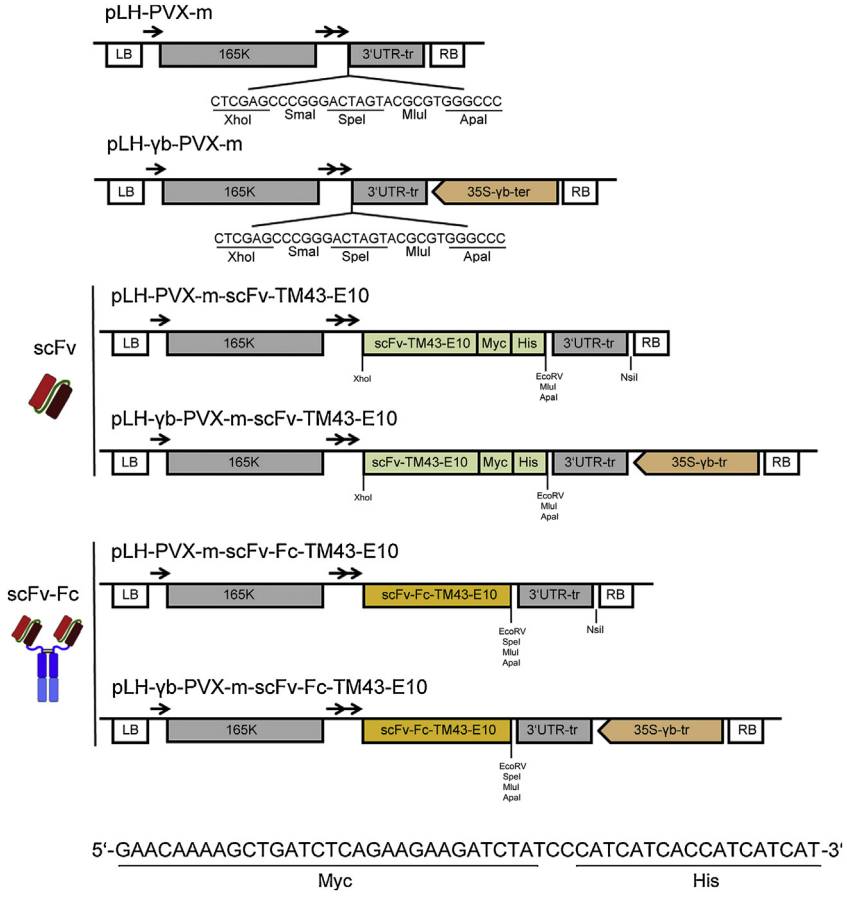

Fig. 1. Schematic representation of the PVX-based transient expression vectors. Features are as follows: 165 K, RNA dependent RNA polymerase; 3′-UTR-tr, 3′ non-translated region of PVX and terminator signal; 35S-γb-ter, expression cassette of PSLV γb silencing suppressor; scFv-TM43-E10, scFv-Fc-TM43-E10, recombinant antibody encoding sequences; Myc, Myc-tag; His, His-tag; single arrow, 35S promoter; double arrow, subgenomic RNA promoters. The bottom field represents the Myc-tag and His-tag sequences that were linked to the C-terminal end of the scFv-TM43-E10 antibody fragment. The location of restriction sites (*Xba*I, *Sma*I, *Spe*I, *Mlu*I, *EcoR*V, *Nsi*I and *Apa*I), left (LB) and right (RB) borders are also shown.

These expression vectors contain between the left and right T-DNA borders the CaMV 35S promoter, the RNA-dependent RNA polymerase gene of PVX, double subgenomic promoter (TGB1 and coat protein subgenomic promoters), 3′ nontranslated region of PVX and terminator. The pLH-γb-PVX-m vector includes an additional expression cassette for PSLV γb gene silencing suppressor. The scFv-TM43-E10 protein contains C-terminal His-tag for affinity purification and Myc-tag for detection by Western blot.

Four final transient expression vectors were transformed into *A. tumefaciens* and delivered by agroinfiltration into middle leaves of six-week old *N. benthamiana* plants (Figure S1). To evaluate the effect of the PSLV γb gene silencing suppressor on scFv-TM43-E10 and scFv-Fc-TM43-E10 accumulation agroinfiltrated *N. benthamiana* leaf samples were harvested at 5 dpi and investigated by qPCR and Western blot analysis. Plant probes were first examined for the presence of γb mRNA (Figure S2). RT-PCR analysis with γb specific primers confirmed the presence of γb mRNA in the pLH-γb-PVX-m agroinfiltrated leaves. There was not any PCR product in the pLH-PVX-m samples. An actin gene selected as an internal control confirmed the presence of cDNA in all investigated samples.

The mRNA expression of target genes was studied in agro-infiltrated samples by qPCR using TM43-E10 specific primers and *cyp* gene as an internal control. Co-expression of γb silencing suppressor resulted in higher mRNA accumulation level by up to 4.1 and 17 fold for scFv-TM43-E10 and scFv-Fc-TM43-E10, respectively, when compared with plants infiltrated with the pLH-PVX-m vector (Fig. 2a). The expression of recombinant antibody fragments was verified at protein level by immunoblotting. This analysis demonstrated the presence of the protein bands of expected molecular size for scFv-TM43-E10 and scFv-Fc-TM43-E10 in leaf extracts (Fig. 2b).

To confirm that the elevated mRNA levels correlated with the increased protein accumulation, the levels of scFv-TM43-E10 and scFv-Fc-TM43-E10 antibody fragments were measured using ELISA in plant tissue. The pLH-γb-PVX-m expression vector resulted in higher protein yields in comparison to the pLH-PVX-m vector for both antibody formats. As illustrated in Fig. 3 about 41 μg/g fresh leaf weight for the pLH-PVX-m-scFv-TM43-E10 agroinfiltrated leaves and 82.5 μg/g fresh weight for the pLH-γb-PVX-m-scFv-TM43-E10 samples were detected. The same tendency was observed for the scFv-Fc-TM43-E10 antibody fragment: pLH-γb-PVX-m-scFv-Fc-TM43-E10 yielded 9.7 μg/g fresh leaf weight compared to the pLH-PVX-m-scFv-Fc-TM43-E10, which yielded 45.9 μg/g fresh leaf weight.

The accumulation of recombinant scFv-TM43-E10 and scFv-Fc-TM43-E10 proteins was further monitored in a time course experiment. *N. benthamiana* leaves agroinfiltrated with both pLH-PVX-m and pLH-γb-PVX-m vectors carrying scFv and scFv-Fc antibody fragments were collected from three independent plants on days 2, 5, 7 and 10 and expression was assayed by immunoblot. The experiment was repeated twice. Fig. 4 shows the results of a typical experiment. This analysis indicated that the expression profile of individual proteins was similar irrespective of the expression vector. The accumulation of scFv-TM43-E10 and scFv-Fc-TM43-E10 recombinant antibodies increased steadily from day 2, peaked between days 5 and 7 and declined at 10 dpi. Temporal analysis of recombinant protein expression from pLH-PVX-m and pLH-γb-PVX-m vectors revealed that both investigated antibody fragments displayed the highest expression level at 5–7 dpi. Therefore, we selected this time point to take plant material for protein extraction.

3.2. Characterization of plant produced antibodies

Purification of scFv-TM43-E10 antibody fragment was performed by IMAC based on C-terminal His-tag. The scFv-Fc version of TM43-E10 antibody was purified from leaf extracts via Protein A affinity chromatography. SDS-PAGE analysis of the eluted fractions after affinity chromatography revealed that scFv-TM43-E10 and scFv-Fc-TM43-E10 proteins were selectively enriched from leaf extracts. Samples derived from the first purification step were separated by size exclusion chromatography. Reducing SDS-PAGE analysis of the purified antibodies demonstrated single bands at

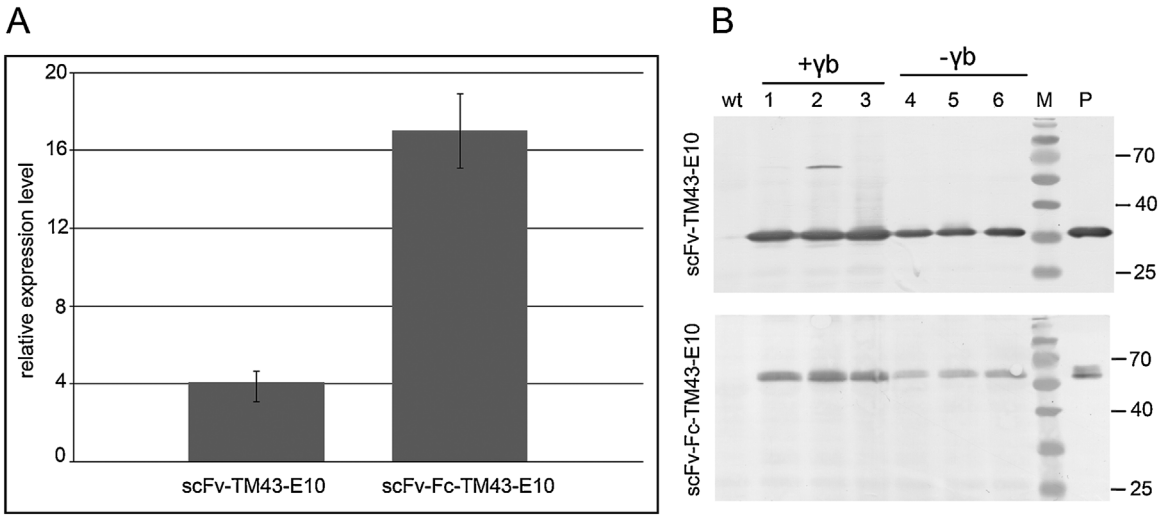

Fig. 2. Transient expression of scFv-TM43-E10 and scFv-Fc-TM43-E10 recombinant antibodies. *N. benthamiana* plants were agroinfiltrated with pLH-PVX-m and pLH-γb-PVX-m expression vectors harboring scFv-TM43-E10 and scFv-Fc-TM43-E10 sequences. RNA and protein samples were collected at 5 dpi and investigated by RT-PCR and Western blot.

Quantification of mRNA level of target genes by qPCR (a). Total RNA from agroinfiltrated leaf samples was subjected to cDNA synthesis and subsequent PCR analysis using TM43-E10 specific primers. mRNA levels were normalized to *cyp* gene. Results are presented as fold induction relative to mRNA levels in pLH-PVX-m agroinfiltrated samples. Each sample was pooled from 3 infiltrated leaves of one plant. The experiments were repeated 3 times. Each column represents mean and standard deviation (n = 6).

Western blot analysis (b). Leaf protein extracts (12 μg of TSP) were separated on reduced 12% SDS-PAGE gel and probed with anti-c-Myc (scFv-TM43-E10) and anti-human IgG (Fc) (scFv-Fc-TM43-E10) antibody. Each lane represents a pooled sample created by mixing three infiltrates sports of one plant. Three pooled samples are shown for each expression vector. Protein preparations extracted from HEK293-6E, *E. coli* cultures (200 ng) and mock inoculated plants were used as positive and negative controls, respectively.

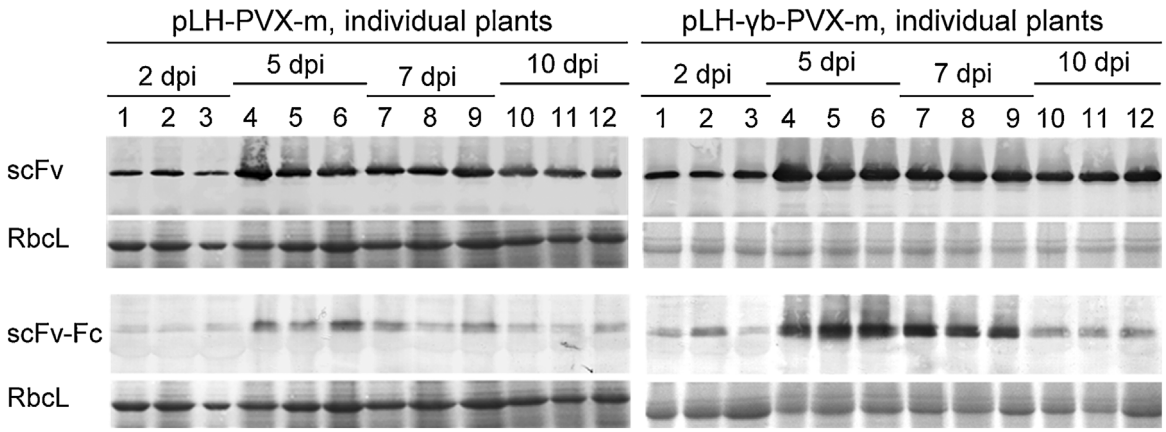

Fig. 3. Transient expression of scFv-TM43-E10 and scFv-Fc-TM43-E10 recombinant antibodies in *N. benthamiana* leaves over a 10 day time course. 12 μg of total proteins extracted from agroinfiltrated leaves taken on 3, 5, 7 and 10 dpi were separated on reduced 12% SDS-PAGE and analysed for the presence of scFv-TM43-E10 and scFv-Fc-TM43-E10 proteins by immunoblot. Each lane represents a pooled sample generated by combining three infiltrated sports from one plant. Three samples taken from independent plants are shown for each time point. The upper panels show scFv-TM43-E10 (scFv) and scFv-Fc-TM43-E10 (scFv-Fc) antibody fragments. The bottom panels show Coomassie-stained Large Rubisco subunit (RbcL) as a loading control.

approximately 28 kDa and 50 kDa for scFv-TM43-E10 and scFv-Fc-TM43-E10, respectively (Fig. 6).

The functionality of plant-derived antibody fragments was determined by ELISA. To this end ELISA plates were coated with OmpD recombinant protein and probed with different dilutions of purified antibody fragments. As shown in Fig. 6a plant-produced antibodies were able to recognize the OmpD antigen. The binding to OmpD protein increased with the concentration of scFv-TM43-E10 and scFv-Fc-TM43-E10 proteins in the reaction. The antigen binding activity of plant-derived antibodies was further compared with the scFv-TM43-E10 produced in bacterial cells. The scFv-Fc-TM43-E10 format extracted from mammalian cell culture served as a reference for the scFv-Fc derivative isolated from plant tissue. As expected the scFv-TM43-E10 preparations from both plant material and bacterial cells exhibited similar binding properties. The sensitivity of scFv-Fc-TM43-E10 plantibody to OmpD was also comparable to that of the scFv-Fc format generated in mammalian cells.

To determine the antigen detection limit ELISA plates were coated with different concentrations of OmpD protein ranging from 0.1 ng/ml to 1000 ng/ml and probed with purified plant-produced antibody fragments at a half maximal saturation. The results of this assay are illustrated in Fig. 6b. The minimal OmpD concentration that has been detected by scFv-TM43-E10 proteins was 50 ng/ml. The antigen detection limit of the scFv-Fc-TM43-E10 antibody fragment was about 20 ng/ml.

The plant-made scFv-TM43-E10 and scFv-Fc-TM43-E10 antibodies were tested with extracts from salad leaves infected with S. Typhimurium. This analysis demonstrated that plant-produced diagnostic antibodies could recognize the OmpD antigen in all infected samples investigated (Fig. 7).

Overall, our results demonstrate that the PVX-based vector enhanced with the γb silencing suppressor was able to direct the plant production of scFv-TM43-E10 and scFv-Fc-TM43-E10 diagnostic antibody fragments similar in antigen binding activity to the controls generated in bacterial and mammalian cells.

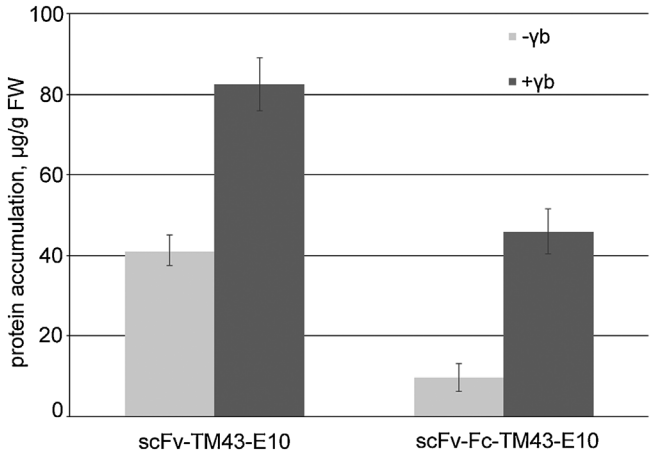

Fig. 4. Accumulation of the antibody fragments. *N. benthamiana* leaves were agroinfiltrated with pLH-PVX-m and pLH-γb-PVX-m expression vectors carrying scFv-TM43-E10 and scFv-Fc-TM43-E10 sequences, collected at 5 dpi and subjected to ELISA to estimate protein accumulation level. Each sample represents pooled sample created by mixing three leaf samples of one plant. Values correspond to the mean accumulation level and standard deviation obtained from three independent adroinfiltration experiments.

4. Discussion

The transient expression exploiting Agrobacterium-mediated delivery of expression vectors has become the preferred plant-based platform due to its advantage in speed, yield of recombinant proteins and the reduced concern for transgene escape. Transient recombinant protein production depends on both the expression vector and the plant host. Currently two types of expression vectors, non-viral or plant virus-based, can be used for transient protein production. Expression vectors utilizing a plastocyanin promoter [37] and the 5′- and 3′- translated region of CPMV [38] are the most efficient non-viral systems described in the literature. The latest development in plant virus vectors is the vector deconstruction strategy. Deconstructed viral vectors based on TMV [18], CPMV [39], PVX [21] and BeYDV [40] provide high protein yield through a reduction to components essential for recombinant protein production. In comparison to a deleted PVX vector developed by Giritch et al. [21], the PVX vector described in this work uses the fused TGB1 and coat protein subgenomic promoter for foreign gene expression and is completely deficient in cell-to-cell and long distance movement. We have taken advantage of Agrobacterium-mediated delivery of an expression cassette controlled by the 35S promoter. Infiltration of *N. benthamiana* leaves with pLH-PVX-m and pLH-γb-PVX-m vectors carrying scFv and scFv-Fc formats of a TM43-E10 antibody resulted in accumulation of mRNA and proteins as was evidenced by qPCR, Western blot analysis and ELISA.

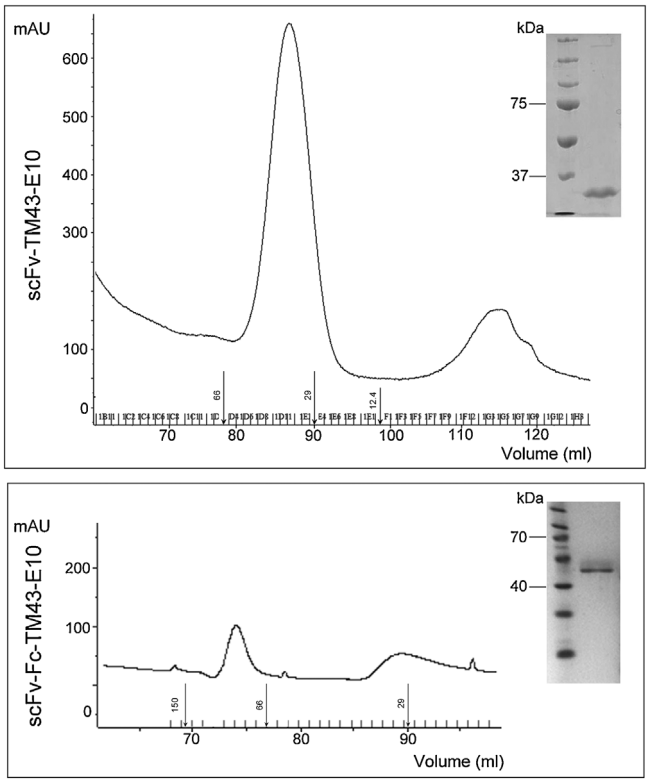

Fig. 5. Characterization of plant-derived antibodies by size exclusion chromatography and subsequent SDS-PAGE gel electrophoresis. The Superdex 200-16/60 column (GE Healthcare) was calibrated with Cytochrom C (12.4 kDa), Carbonic Anhydrase (29 kDa), Albumin (66 kDa) and Alcohol Dehydrogenase (150 kDa). Purified proteins were separated on reduced 12% SDS-PAGE gel and stained with Coomassie blue.

One of the factors reducing the efficiency of transient expression is mRNA degradation by RNA silencing. The gene silencing is involved in regulating expression of endogenous genes as well as reducing or eliminating the effects of invading pathogens such as viruses [41,42]. To neutralize this silencing response and enhance the level of foreign gene expression, co-expression of proteins that are capable to interfere with components of this resistance is used routinely. These factors are known as "suppressors" of RNA silencing. So far, several suppressors of RNA silencing have been evaluated for their ability to counteract gene silencing and enhance foreign gene expression in transient assay, including p19 from either *Artichoke mottled crinkle virus* (AMCV) [43] or *Tomato bushy stunt virus* (TBSV) [44], HcPro from *Potato virus* Y (PVY) [37], p25 from PVX [45] and γb from *Barley stripe mosaic virus* (BSMV) [46]. We investigated the influence of the γb protein from *Poa semilatent virus* (PSLV) on foreign protein accumulation in a transient assay. PSLV is a plus-strand RNA virus of the genus *Hordeivirus* with a tripartite genome consisting of RNAα, RNAβ and RNAγ. RNAα encodes a component of viral replicase. RNA β encodes coat protein and three movement proteins. RNAγ is bicistronic and encode the other component of viral replicase and a non-structural γb protein [47,48]. γb contributes to symptom severity, systemic viral movement and RNA silencing suppression [33,49]. Although the anti-silencing activity of PSLV γb protein has been shown a decade ago [33], the mechanism of γb action has not been investigated. Several lines of evidence indicate that silencing suppressor properties of γb may be mediated by its RNA binding activities. The γb protein likely binds in a sequence unspecific manner to ssRNA via a coiled-coil domain with zinc binding sites, to prevent RNA degradation by RNA-induced silencing complex [33,50]. In this study we demonstrate for the first time the effectiveness of γb to increase the recombinant protein expression level. The increased transcription of *scFv-TM43-E10* and *scFv-Fc-TM43-E10* genes was detected by qPCR analysis in *N. benthamiana* leaves agroinfiltrated with pLH-γb-PVX-m vector when compared to pLH-PVX-m expression vector. The mRNA expression levels correlated with protein yields as was quantified by ELISA. Application of the γb silencing suppressor caused two and five fold increases in scFv-TM43-E10 and scFv-Fc-TM43-E10 accumulation, respectively.

The most efficient silencing inhibitor described to date is the p19 protein. In general, accumulation enhancement by the p19 silencing suppressor was dependent on the specific protein and ranged between 2–50 fold. The highest increase in expression level, approximately 50-fold, has been documented for GFP protein [51]. Expression of mAbH10 [43] and non-structural HIV-1 protein Nef [45] has only been enhanced by 10 and 4.4 fold, respectively. The amount of hEGF recovered from samples co-infiltrated with p19 was 3.9 fold higher than that from the samples without p19 [52].

Four plant viral silencing suppressors, namely the *Tobacco etch virus* (TEV)-encoded P1/HC-Pro, the BSMV-encoded γb, the TBSV-encoded P19 and the *Sugarcane bacilliform virus* (SCBV)-encoded OrfI, were evaluated for their ability to enhance the expression of *eYFP* and *gus* genes in young leaf segments and protoplasts of sugarcane [46]. Among these suppressors TEV p19 and BSMV γb proteins were the most efficient. Complementation experiments revealed functional similarity of the BSMV and PSLV γb proteins [33].

The amount of recombinant proteins expressed in agroinfiltrated *N. benthamiana* leaves as measured by ELISA was 82.5 μg/g fresh weight for scFv-TM43-E10 and 45.9 μg/g fresh weight for scFv-Fc-TM43-E10. These accumulation levels are not superior to protein expression levels shown for the movement-competent deconstructed virus vectors [53]. However, the yields are comparable to the expression levels described for movement-deficient virus vectors and non-viral vectors [54]. Our transient expression system might be improved by directing scFv-Fc-TM43-E10 recombinant antibody into various cell compartments, particularly endoplasmatic reticulum (ER). Several studies demonstrated that the addition of a KDEL peptide to recombinant proteins has allowed efficient ER targeting and increased their accumulation in 2–10 times [37,55,56].

The expression profile in transient assay may be different for each product expressed. In most cases maximal expression levels occur at 3–5 days after infiltration and fade rapidly after 7–9 days. For example, the maximal accumulation for GFP, P24 and SARS-CoV proteins has been observed at 3 dpi [51,57]. The highest expression level for L2 and non-structural HIV-1 Nef proteins was reached at 5 dpi and 9 dpi, respectively [45,57]. In our study scFv and scFv-Fc formats of TM43-E10 antibody displayed a similar expression profile with highest accumulation level at 5–7 dpi.

The functionality of the recombinant proteins purified from agroinfiltrated leaves was assessed by titration ELISA. This analysis indicated that scFv-TM43-E10 and scFv-Fc-TM43-E10 antibody fragments bound to OmpD antigen provided as purified protein or probes prepared from infected plant leaves confirming feasibility of plants as expression platform for S. Typhimurium diagnostic antibodies. The minimal concentration of OmpD protein that can be detected by scFv-TM43-E10, scFab-TM43-E10 antibody fragments was 50 ng/ml. About 20 ng/ml of antigen were detected by scFv-Fc-TM43-E10.

The biological activity of plant-generated antibodies is usually compared to that of reference antibodies produced in bacterial or animal cell expression systems. Therefore, the scFv-TM43-E10 and scFv-Fc-TM43-E10 antibody derivatives have been also expressed in bacterial and mammalian cell cultures and included into the

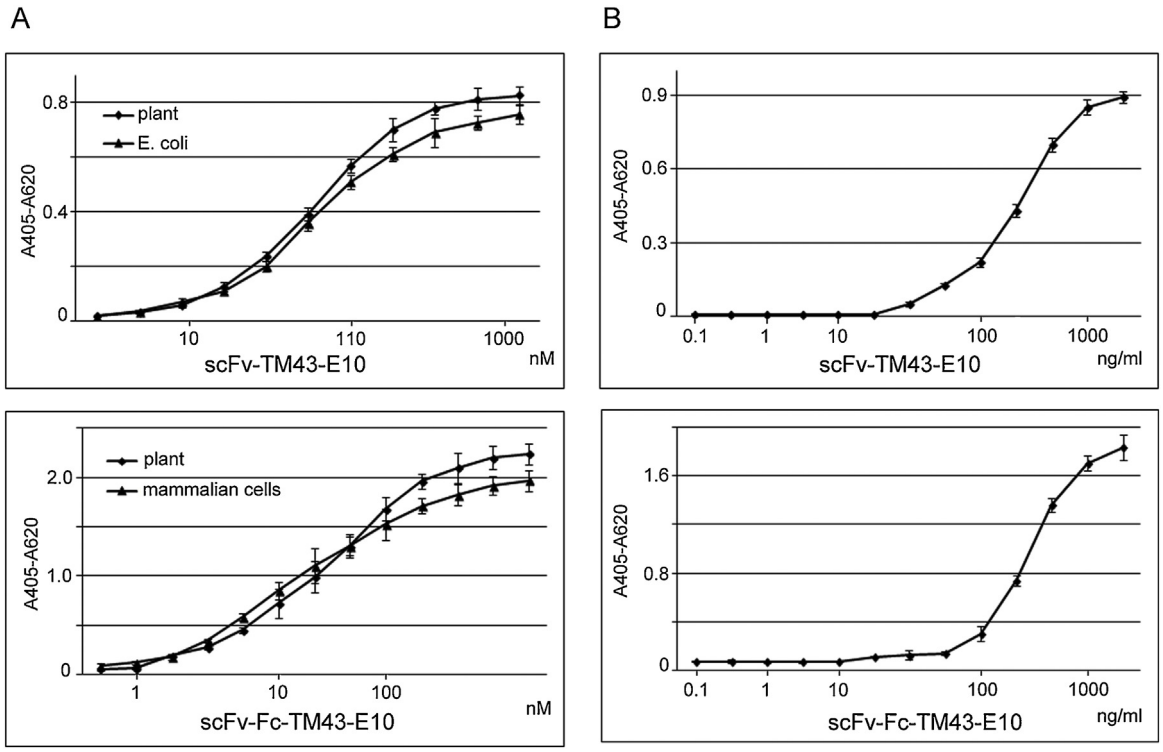

Fig. 6. Functional characterization of plant-derived *S.* Typhimurium diagnostic antibodies. Antibody titration ELISA (A). ELISA plates were coated with 1 μg/well OmpD protein and incubated with serial dilutions of recombinant antibodies. Plant-produced, *E. coli* (scFv-TM43-E10) and mammalian cells-derived (scFv-Fc-TM43-E10) proteins were involved in this assay.
Antigen titration ELISA (B). Different dilutions of OmpD protein (0.1 ng/ml-1000 ng/ml) were coated to the wells. Antigen detection was performed with the scFv-TM43-E10 and scFv-Fc-TM43-E10 recombinant antibodies purified from plant material at a concentration of half maximal saturation. Data are shown as mean and standard deviation. Results are a combination of two independent experiments (n = 4).

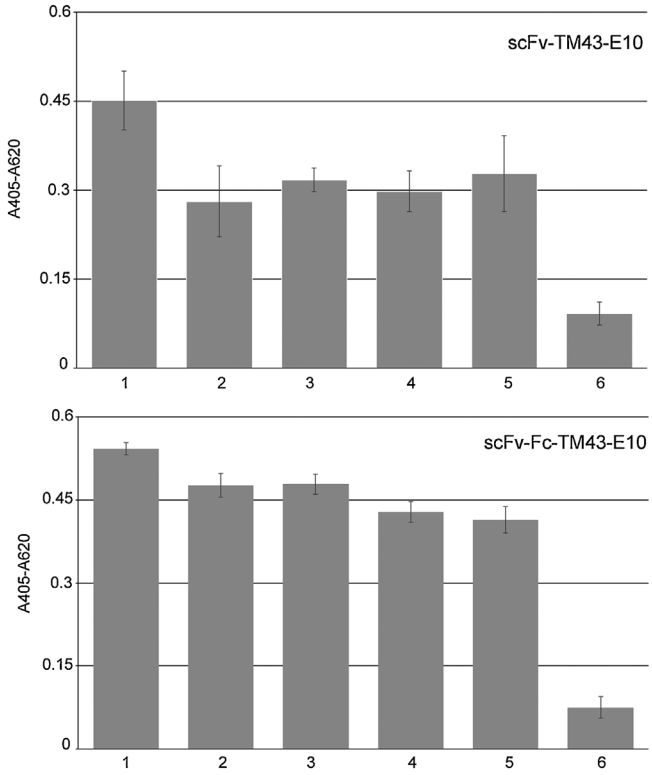

Fig. 7. ELISA test of scFv-TM43-E10 and scFv-Fc-TM43-E10 antibody fragments. ELISA plates were coated with S. Typhimurium suspension (lane 1), protein extracts from S. Typhimurium infected (lanes 2–5) and non-infected (lane 6) salad leaf tissue. 400 ng/well for scFv-TM43-E10 and 150 ng/well for scFv-Fc-TM43-E10 were added for detection of OmpD antigen. Values represent the means with standard deviation from two independent tests.

functional test. The antigen binding of plant-derived scFv-TM43-E10 antibodies was similar to scFv-TM43-E10 recombinant proteins isolated from *E. coli*. Furthermore functional ELISA test also showed a similar functional activity for plant and mammalian cell culture-made scFv-Fc-TM43-E10 antibody fragments (Fig. 5A). Our data are in agreement with findings described in several articles with respect to the functional equivalence of the recombinant proteins produced in different expression systems. For example plant-derived 6D8 and hE16 mAbs demonstrated similar to mammalian cell-derived counterpart antigen specificity [58]. Yuan et al. [59] reported that the biological activity of antizearalenone scFv "plantibody" was comparable to that of a bacterially produced scFv antibody. A number of research groups investigated the binding properties of the plant-derived anti-HIV VRC01, 2G12 and b12 antibodies in comparison to reference counterparts [60–62]. These studies also revealed functional equivalence between plant- and mammalian cells-made recombinant proteins.

In summary, we produced diagnostic scFv-TM43-E10 and scFv-Fc-TM43-E10 antibodies by means of transient expression in plants. The accumulation of recombinant proteins was enhanced by exploring the PSLV γb silencing suppressor demonstrating for the first time its applicability for biotechnological purposes. Plant-derived scFv and scFv-Fc formats of TM43-E10 protein were biologically active as demonstrated by ELISA suggesting the feasibility of the *N. benthamiana* production platform for expression of diagnostic *S.* Typhimurium antibodies.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.btre.2019.e00314.

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