



Production of active single-chain antibodies in seeds using trimeric polyoleosin fusion

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ABSTRACT

A variety of single-chain variable fragments (scFv) that had been previously developed to the surface epitopes of infective *Trichostrongylus colubriformis* L3 pathogenic gut nematodes of sheep were fused to a trimeric version of polyoleosin (three head-to-tail repeats of oleosin) and expressed *in planta* under the control of an Arabidopsis oleosin promoter. The fusion products were found to accumulate in oil bodies (OBs) at the range of 0.25–0.9% of the total seed protein which is comparable with the main 18 kDa isoform of Arabidopsis seed oleosin. Immunofluorescence microscopy and immuno-binding were used to demonstrate that it is possible to both purify the recombinant protein via enrichment for OBs as well as use the OBs emulsion to deliver functional recombinant scFv. This work presents a novel fusion strategy platform to boost the productivity and simplify the delivery of recombinant single chain antibodies and other like proteins.

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1. Introduction

During the last few years, smaller but functional antibody fragments including: monovalent antigen binding fragments (Fab); single chain variable fragments (scFv); and single-domain antibodies (also known as sdAb, NanobodiesTM, variable fragment of heavy-chain antibodies or VHH) have been produced more economically and marketed as alternative products for a number of applications ranging from simple research tools to highly refined biopharmaceutical drugs (Holliger and Hudson, 2005). A critical requirement for the expression of full-length antibodies and Fab is the correct folding and appropriate self-assembly of the individual light and heavy chains. Therefore, while prokaryotic expression systems are relatively easy to scale up, they have to be optimised to prevent proteolysis, tagging to other proteins to improve protein folding and/or disulfide bond formation, and to express the correct proportion of light and heavy chains in order to achieve increased titres (Andersen and Reilly, 2004).

ScFv are an engineered antibody created directly from sub-cloning of heavy and light chains derived from a hybridoma or by panning a phage display library. Compared to Fab, scFv are only half the size, yet retain similar binding specificity of the parent immunoglobulin. In various preclinical cancer models, scFv have

gained popularity in recent years as the targeting agent of choice over traditional agents, such as monoclonal antibodies, owing to factors such as a low degree of immunogenicity, pharmacokinetics and bio-distribution profiles (Cheng and Allen, 2010). Even smaller than scFv, the isolated VHH from the Camelidae family possess a high conformational stability and are stable and active in harsh conditions, such as: detergents (Dolk et al., 2005) and the digestive tract (for review see Dumoulin et al., 2002; Harmsen and De, 2007; Winichayakul et al., 2009). The fact that scFv and VHH are relatively small greatly simplifies the cloning and expression systems needed to produce a functional antibody (Fischer et al., 1999; Ghahroudi et al., 1997).

However, although their simplified production is very attractive, the smaller antibody fragments tend to exhibit a shorter circulatory half-life than full-length antibodies and this can become a limitation for therapeutic use unless frequent dosing is provided (Andersen and Reilly, 2004). A large number of studies have established that plants can efficiently produce high levels of antibody fragments (for reviews see Liénard et al., 2007; Ma et al., 2003). One of the major advantages of using transgenic plants, over prokaryotic production systems available is the ability of plant cells to perform most post-translational modifications required for appropriate activity, kinetics, stability and optimal solubility (Gomord and Faye, 2004). Plant-based production systems possess several advantages over traditional mammalian based production systems in that cultivation of mammalian cells can be difficult and expensive media and equipment are required, particularly in the case

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of production immunofluorescence microscopy of pharmaceutical recombinant proteins. Plants are also less likely to contain animal pathogens such as human viruses or deleterious contaminants such as prions (Capuano et al., 2007). Orally and topically applied antibody fragments known as passive vaccines are another intense area of research in plant biopharming (Arntzen, 2005) and to ensure competitive production costs both the level of protein accumulated as well as the delivery schemes have been improved. In the last two decades, considerable efforts have been made to increase the level of recombinant proteins expressed *in planta* (for reviews see Liénard et al., 2007). This includes increasing the level of transcript by optimising the expressing sequences to the host and targeting the recombinant proteins to specific plant subcellular organelles. Previously, we reported approximately an 8-fold increase in anti-TNF α nanobodyTM accumulation in Arabidopsis plants when targeted to the endoplasmic reticulum (ER) compared to either the chloroplast or vacuole (Winichayakul et al., 2009).

Plant seeds are an attractive organ for recombinant protein production and storage since they typically have a high protein content, low protease activity, and low water content (Ma et al., 2003; Müntz, 1998; Stoger et al., 2000). These factors are important for molecular farming in terms of yield gains as well as convenient storage and transport (Fischer et al., 1999; Tzen et al., 2003). Indeed, recombinant antibodies, vaccine antigens and other recombinant proteins have been shown to accumulate at high levels in seeds and remain stable and functional for years at ambient temperature (Nochi et al., 2007; Schunmann et al., 2002; Stoger et al., 2005). In some instances it could also be envisaged that seeds could be directly consumed, allowing oral delivery of pharmaceutical proteins for passive immunisation and treatment of certain diseases (Daniell et al., 2001; Lamphear et al., 2002; Takagi et al., 2005). For example, pea seeds expressing Eimeria-specific antibodies was used as fodder in feeding trials and found to prevent Coccidiosis, a diarrheal disease of chickens (Zimmermann et al., 2009).

During the last 15 years, several seed-based production platforms have been developed in a variety of plants, including cereals (Hood et al., 1997; Xue et al., 2003), legumes (Philip et al., 2001), and oilseeds (Moloney et al., 2003). Not only selection of plants but also a wide range of expression strategies have been demonstrated to vary productivity yields; these included the use of specific promoters, subcellular targeting and fusion affinity tags that facilitate protein purification or locating recombinant proteins to seed oil bodies (OBs) by oleosin fusion (Hwang et al., 2002; Nykiforuk et al., 2005). OBs are organelles located in the cells of plant seeds and provide a source of energy for seed germination. OBs are delineated by a phospholipid monolayer (derived from the ER) which in turn is encapsulated within a layer of protein oleosin (Tzen et al., 2003). The peculiar topology of oleosin is attributed to its physical properties which includes a folded highly conserved hydrophobic core being embedded into the triacylglyceride matrix whereas the flanking hydrophilic arms (both N- or C-terminal domains, and any fused proteins) are exposed to the cytosol on the surface of the OB (Fig. 1, Hwang et al., 2002; Roberts et al., 2008). Oleosin fused functional, recombinant proteins including β -glucuronidase (GUS), hirudin, xylanase, and insulin have been successfully expressed in transgenic plant seeds (Deckers et al., 2003; Lee et al., 1991; Nykiforuk et al., 2005; Parmenter et al., 1995). Oleosin fusion technology addresses the important issue of purification by way of reducing productivity costs (Ma et al., 2003) since they have the potential to accumulate up to 3% of total soluble protein in the seed (Roberts et al., 2008; Tzen et al., 2003) and can be easily separated from the other seed cell components by flotation centrifugation, taking advantage of their very low density (Parmenter et al., 1995). In addition, purified OBs can be rapidly formulated since oleosin is a natural emulsificant which forms a very stable oil-in-water emulsion. Thus the properties of oleosin enable recombinant protein

production as well as a relatively simple purification and/or incorporation of the protein as a micro-particle into dietary supplements (Deckers et al., 2003).

Linking multiple units of oleosins by either chemical cross linking (Peng et al., 2003) or by protein engineering fusing oleosin (up to six) repeats in a head-to-tail arrangement (polyoleosin) (Scott et al., 2007, 2010) has been shown to greatly enhance OB integrity at elevated temperatures and in the presence of proteases. Furthermore, fusion of GFP to dimeric or trimeric forms of oleosins resulted in an increase in the yield compared to fusions with single oleosins due to enhanced protein stability (Banilas et al., 2011). The attributes of polyoleosin conferring higher OB integrity and enhanced fused protein stability led us to evaluate the potential to both produce and deliver functional scFv. We selected three previously characterised anti-CarLA scFv clones: Tc.C1, Tc.C2, and Tc.C3 (Maass et al., 2009) as representatives of the three main epitope groups of the glycolipid antigen CarLA (Carbohydrate Larvae Antigen). CarLA is a molecule found on the surface of the infective L3 stage of many intestinal nematode parasite species including *Trichostrongylus colubriformis*; protective anti-CarLA IgA are naturally produced by the host once immunity has developed (Harrison et al., 2008). The accumulation of polyoleosin anti-CarLA scFv fusion clones *in planta*, as well as their functional ability to bind to the surface of nematodes was determined and the possibility of adapting this technology to improve the productivity of specific recombinant scFv proteins in agricultural plants is discussed.

2. Materials and methods

2.1. Construct design

Coding sequences of anti-CarLA Tc.C1, Tc.C2, or Tc.C3 were optimised for *in planta* expression and fused individually to either the N- or C-terminus of trimeric polyoleosin (Fig. 1). The trimeric form of polyoleosin was preferred over the hexameric form since the latter was found to delay seed germination (Scott et al., 2010). All coding sequences were optimised for the expression in thale cress (*Arabidopsis thaliana*) and custom synthesised by GENEART AG (Regensburg, Germany). The nucleotide sequences were made to contain in-frame 5' *Ascl*- and 3' *NotI*-flanking sites (Supplementary Fig. S1a). In addition the 304 bp Arabidopsis ubiquitin 10 (UBQ10) intron sequences (Norris et al., 1993) was inserted into the coding sequences (Supplementary Fig. S1a) to enhance expression *in planta* (Rose, 2004). The position of intron insertion was checked for correct splicing using Splice Predictor online (<http://deepc2.psi.iastate.edu/cgi-bin/sp.cgi>) and NetGene2 Server (<http://www.cbs.dtu.dk/services/NetGene2/>). The synthetic sequences of trimeric sesame polyoleosin contained in-frame 5' *EcoRI*- and 3' *XbaI*-flanking sites while the first sesame oleosin repeat contained the UBQ10 intron (Supplementary Fig. S1b).

Supplementary material related to this article found, in the online version, at <http://dx.doi.org/10.1016/j.jbiotec.2012.07.195>.

To generate the C-terminal trimeric *myc*-tagged trimeric polyoleosin (3 $\times$ OLE::3 $\times$ *myc*) and the scFv fusion (anti-CarLA Tc.C::3 $\times$ OLE::3 $\times$ *myc*, Fig. 1a), the construct was designed and synthesised with attL (GATEWAYTM) flanked donor cassettes (Supplementary Fig. S1c). This construct contained a modified Kozak sequence (Kozak, 1981) before the ATG translation start site; unique *Ascl*, *EcoRI*, *NotI* and *XbaI* cloning sites, three repeat *myc* epitopes, and a tetranucleotide stop codon (Brown et al., 1990). The synthetic constructs of trimeric polyoleosin (Supplementary Fig. S1b) and anti-CarLA scFv (Supplementary Fig. S1a) were processed by restriction digestion and ligation to consequently produce a series of attL flanked expression cassettes as shown in Supplementary Fig. S1e and f.

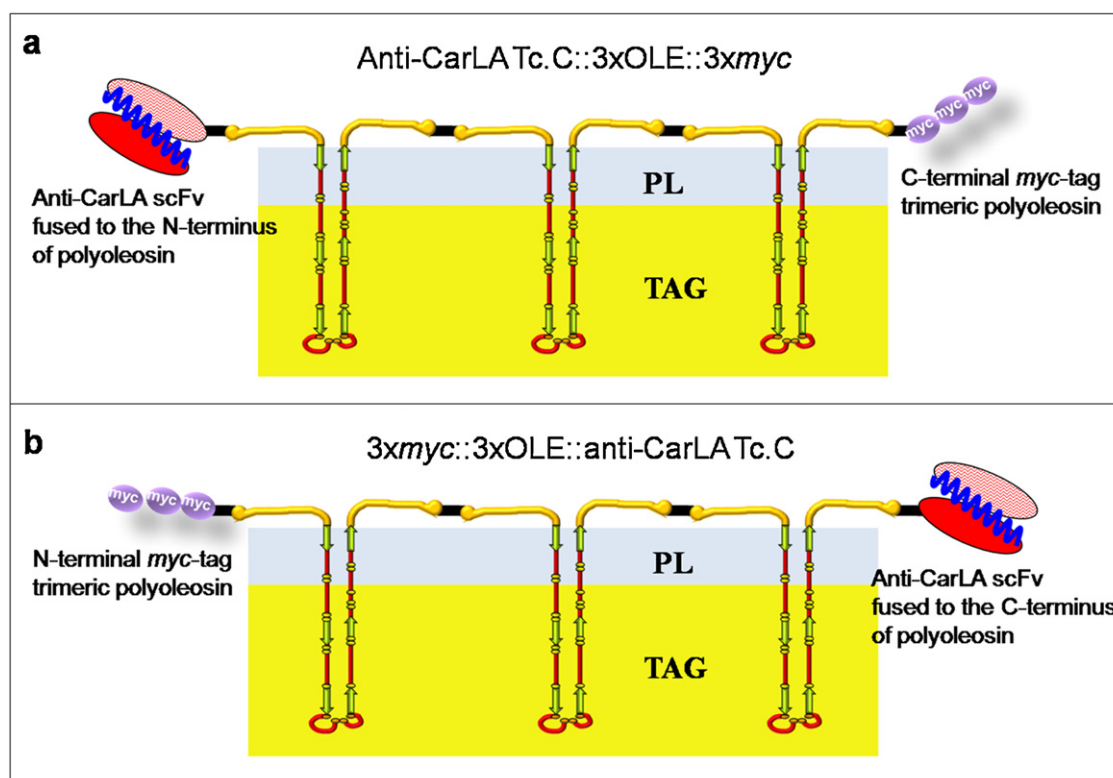


Fig. 1. Schematic images of anti-CarLA scFv–polyoleosin fusions. (a) Anti-CarLA Tc.C::3 × OLE::3 × myc fusions are designations for the expression cassettes that have anti-CarLA scFv either Tc.C1, Tc.C2, or Tc.C3 fused to the N-terminus of trimeric polyoleosin and tagged with triple c-myc at the C-terminus. (b) 3 × myc::3 × OLE::anti-CarLA Tc.C fusions are designations for the expression cassettes arranged in reverse orientation to (a). The image also demonstrated a predicted structural topology of oleosin hydrophobic core being embedded into the triacylglyceride (TAG) matrix delineated by phospholipid (PL) monolayer to form a discrete OB whereas the flanking hydrophilic N- and C-terminal arms and any fused proteins are exposed to the cytosol.

To generate the N-terminal trimeric myc-tagged trimeric polyoleosin (3 × myc::3 × OLE) with the C-terminal scFv fusion (3 × myc::3 × OLE::anti-CarLA Tc.C, Fig. 1b), the construct as shown in Supplementary Fig. S1d was designed and synthesised with attL (GATEWAY™) flanked donor cassettes. This construct contained a modified Kozak sequence (Kozak, 1981) before the ATG translation start site followed by three repeated c-myc epitopes with intron insertion, unique *Ascl*, *EcoRI*, *NotI* and *XbaI* cloning sites and a tetranucleotide stop codon (Brown et al., 1990). The synthetic constructs of trimeric polyoleosin (Supplementary Fig. S1b) and anti-CarLA scFv (Supplementary Fig. S1a) were processed by restriction digestion and ligation to consequently produce a series of attL flanked expression cassettes as shown in Supplementary Fig. S1g and h.

The attL sites allowed the use of the LR reaction to insert each complete cassette into the GATEWAY™ adapted plant binary vector named pKOLEp. This vector was modified from previously used binary vectors described in Winichayakul et al. (2008) to contain a GATEWAY destination cassette under the control of the Arabidopsis oleosin promoter (Plant et al., 1994) and octopine synthase 3' terminator, and *NPTII* gene for kanamycin resistant selection under the NOS promoter and the NOS terminator.

2.2. Expression in *A. thaliana* and selection of transgenic lines

The floral-drop method (Martinez-Trujillo et al., 2004) was used to transform the *A. thaliana* Columbia ecotype oleosin deletion mutant (*Ole1*, SALK T-DNA insertion line SM.3.29875) by *Agrobacterium tumefaciens* strain GV3101 containing the binary constructs. This line was chosen on the assumption that the absence of the predominant native oleosin (At4g25140) would lead to

an elevated level of recombinant polyoleosin-scFv accumulation (Moloney et al., 2003). Transgenic seeds were grown for 2 weeks on half-strength Murashige-Skoog media containing 1% (w/v) sucrose and 50 mg/l kanamycin at 22–24 °C under continuous light. The healthy greenish seedlings were transferred to soil and grown in a controlled environment at 22 °C with 16 h-daylight. T-DNA insertion was confirmed by PCR using *NPTII* specific primers. Seeds collected from T1 plants were extracted for OBs and analysed by immunoblot for the expression levels of recombinant proteins. Consequently, T2 plants of those lines containing high expression levels of recombinant protein and single copy of T-DNA insertion (confirmed from segregation analysis) were germinated on selection medium and transferred to soil in order to collect sufficient seeds for further protein expression and function analysis.

2.3. SDS-PAGE analysis of polyoleosin and anti-CarLA scFv fusion and immunoblot

Crude OB proteins for SDS-PAGE and immunoblot analysis were prepared with a slight modification to the protocol previously described in Scott et al. (2010). Essentially, 100 mg of plant seeds were homogenised in 1 ml of extraction buffer (0.6M sucrose, 50mM sodium phosphate buffer pH 7.4). The crude OBs were collected as an immiscible fat pad, washed twice with phosphate-buffer-saline (PBS), and re-suspended in 0.3 ml PBS as the final volume. For controls, the native OB-associated proteins were prepared from seeds of wild-type Columbia ecotype or the *ole1* mutant *Arabidopsis* in the same manner.

OB proteins were quantified using both the Invitrogen Quant-iT™ protein assay kit (cat# Q33211) and the Qbit® fluorometer. Known quantities of OB protein preparations were added to

protein gel loading buffer (4 M urea, 50 mM Tris–HCl pH 6.8, 10% glycerol, 1% (w/v) SDS, and a small spatula head of bromophenol blue). One microgram of each protein sample were separated by SDS-PAGE (NuPAGE Novex 4–12% Bis–Tris pre-casted gradient gels from Invitrogen, cat# NP0321) and stained by SilverQuest staining kit (Invitrogen cat#LC6070). For immunoblotting, preparations of 0.25 μ g total OB proteins were separated on precast SDS-polyacrylamide gels (Invitrogen), transferred to a PVDF membrane using the iBlot system (Invitrogen), following the protocol as previously described in Scott et al. (2010). Anti-*c-myc* antibody (produced in rabbit, Sigma cat# C3956) was used as the primary antibody at a dilution of 1/3000; goat anti-rabbit IgG-linked peroxidase (Sigma cat#A6154) was used as secondary antibody at a dilution of 1/10000.

2.4. Immunofluorescence

T. colubriformis L3 larvae (TcL3) were exsheathed followed by purification as described previously in Harrison et al. (2008). Exsheathed TcL3 larvae were pre-treated with 4% milk powder in PBS at 37 °C for 1 h. This was followed by incubation in 1 ml of OBs containing the mixture of anti-CarLA Tc.C1, Tc.C2, and Tc.C3 (from the same group of fusion orientations, by diluting each Tc.C oil-in-water emulsion with PBS to a final concentration of 0.333 μ g/ml) at room temperature with gentle agitation for 1 h. After incubation of the larvae and OBs, the larvae were washed five times with PBS containing 0.1% Tween 20; the larvae were pelleted by centrifugation in a microfuge for 2 min. TcL3 were then labelled with anti-*c-myc* antibody directly conjugated to FITC (Santa Cruz Biotechnology) diluted 1:50 and staining was visualised by fluorescent microscopy (Olympus).

2.5. Immuno-binding assay

Exsheathed TcL3 were blocked with PBS supplemented with 0.05% Tween 20 and 0.2% polyvinylpyrrolidone. Approximately 400 exsheathed and blocked TcL3 were incubated for 1 h with 50 ng of the individual Tc.C scFv–polyoleosin fusion proteins (or in combination) prepared as an oil-in-water emulsion and washed with PBS supplemented with 0.05% Tween 20. Specific binding to whole TcL3 was detected as above protein immunoblotting (Section 2.3)

except that the anti-*c-myc* antibody was diluted 1:1000 and the secondary antibody 1:500. Colour was developed with 3,3',5,5'-tetramethylbenzidine and quantified by measuring the absorbance at 450 nm.

3. Results and discussion

3.1. Accumulation of anti-CarLA scFv–polyoleosin fusions in *Arabidopsis* seeds

In this study, we designed the constructs to allow the polyoleosin (3 $\times$ OLE) to contain both N- and C-terminal fusions, where the fusions consisted of a triple *myc*-tag (3 $\times$ *myc*) at one terminus and an anti-CarLA scFv (anti-CarLA Tc.C) at the other (Fig. 1). Triple tandem *myc* epitopes were used for fast, easy and enhanced detection of fused recombinant proteins (Zhang et al., 2001). Each of the recombinant polyoleosin fusion constructs resulted in the accumulation of the appropriate sized recombinant protein in the OBs. However all constructs with the 3 $\times$ *myc* at the N-terminus (3 $\times$ *myc*::3 $\times$ OLE and 3 $\times$ *myc*::3 $\times$ OLE::anti-CarLA Tc.C either 1, 2 or 3) accumulated at higher levels compared to their counterparts in which the 3 $\times$ *myc* was located at the C-terminus (Fig. 2a). The N-terminal triple *myc*-tag may result in more efficient intron splicing and/or translational efficiency due to the construct design; alternatively the stability of the fusion proteins may depend on which terminus the 3 $\times$ *myc* is attached as noted by Stevens et al. (2001).

Immunoblotting of the 3 $\times$ *myc*::3 $\times$ OLE::anti-CarLA Tc.C fusions (Fig. 2b, lanes 10–12) revealed the presence of additional discreet immunoreactive fragments which did not appear to be present in the samples containing anti-CarLA Tc.C::3 $\times$ OLE::3 $\times$ *myc* fusions (Fig. 2b, lanes 6–8). The apparent absence of these fragments in the samples containing anti-CarLA Tc.C::3 $\times$ OLE::3 $\times$ *myc* may be due to the relatively low proportion of the total protein that the recombinant proteins represented compared to the 3 $\times$ *myc*::3 $\times$ OLE::anti-CarLA Tc.C fusions. These fragments correlate with the predicted sizes of 3 $\times$ *myc*::1 $\times$ oleosin, 3 $\times$ *myc*::2 $\times$ oleosin and 3 $\times$ *myc*::3 $\times$ oleosin (Fig. 2b, arrowed) and were also detected in both 3 $\times$ OLE::3 $\times$ *myc* (Fig. 2b, lane 5) and 3 $\times$ *myc*::3 $\times$ OLE (Fig. 2b, lane 9) control fusions. The fragments were also previously observed by Scott et al. (2010) when trimeric forms of polyoleosin were expressed in

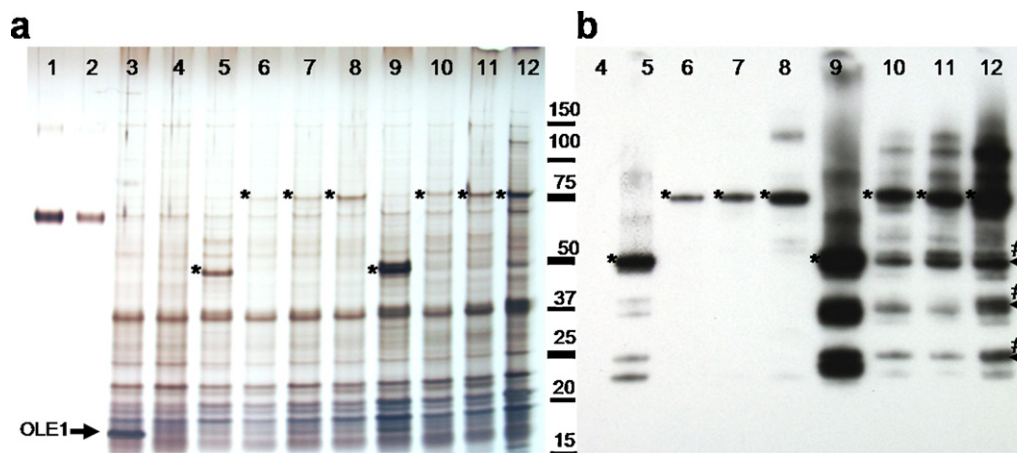


Fig. 2. (a) Anti-CarLA scFv–polyoleosin fused proteins can be observed in SDS/silver stained gels. Lanes 1 and 2 are control loading of BSA standard at 50 and 25 ng, respectively and used for quantitative determination of recombinant proteins. Lanes 3 and 4 are oil emulsion prepared from wild-type (wt) and *ole1* knock-out seeds, respectively, where the arrow indicated the main 18 kDa isoform of oleosin presented in wt but not in the *ole1*. Lanes 5–8 are OB proteins prepared from seeds of 3 $\times$ OLE::3 $\times$ *myc* (5), anti-CarLA Tc.C::3 $\times$ OLE::3 $\times$ *myc* (6, 7, and 8 are Tc.C1, 2, and 3, respectively). Lanes 9–12 are OB proteins prepared from seeds of 3 $\times$ *myc*::3 $\times$ OLE (9), 3 $\times$ *myc*::3 $\times$ OLE::anti-CarLA Tc.C (10, 11, and 12 are Tc.C1, 2, and 3, respectively). Asterisks indicated the expected recombinant proteins at appropriate size which were confirmed by immunoblot analysis (b). Lanes 4–12 of (b) were replicated of (a). Truncated protein fragments detected at the size corresponding to 3 $\times$ *myc*::1 $\times$ OLE, 3 $\times$ *myc*::2 $\times$ OLE, and 3 $\times$ *myc*::3 $\times$ OLE are depicted with arrows numbered 1, 2 and 3.

Arabidopsis OBs and the recombinant proteins were detected using an anti-oleosin antibody. It is possible that some of the fragments represent premature translational termination; in which case only those that had the immune tag would be detected. However, their presence (albeit reduced) in the $3 \times \text{OLE}::3 \times \text{myc}$ control lane indicates the C-terminal tag is present which indicates the translation was complete. Alternatively, proteolytic degradation may be occurring between the oleosin repeats as part of natural mechanism required for oil body mobilisation in which cytosolic proteases control the digestion of OB membrane proteins (either on the N- or C-terminal arms of oleosin) (Tzen and Huang, 1992; Matsui et al., 1999) before lipases or lipoxygenases gain access to the core of the oil bodies (Bhatla et al., 2009).

The accumulation of the full length trimeric polyoleosin and trimeric polyoleosin anti-CarLA scFv(s) fusion in seed OBs was quantified densitometrically from a silver stained gel (using Image J software) and compared either to a BSA standard or to the native 18-kDa oleosin which accounts for 65% of OB-associated proteins and approximately 1.5% of total seed proteins (TSP) (Fig. 2a, Li et al., 2011; Nykiforuk et al., 2005; Siloto et al., 2006). The recombinant $3 \times \text{myc}::3 \times \text{OLE}$ was found to accumulate up to 2.2% of TSP while the $3 \times \text{myc}::3 \times \text{OLE}::\text{anti-CarLA Tc.C}$ (either 1, 2, or 3) levels varied from 0.25 to 0.9% of TSP which is similar to that reported for oleosin–human insulin (Nykiforuk et al., 2005) and oleosin–hIGF-1 (Li et al., 2011) in transgenic seeds. While the total level of recombinant protein accumulated was different in different transgenic lines the full length N- and full length C-terminal anti-CarLA Tc.C3 fusions and their fragments generally accumulated to higher levels than their anti-CarLA Tc.C1 and Tc.C2 fusion counterparts (Fig. 2). At this stage it is not yet known if the differences observed in accumulation are the result of differences in transcription, translation, protein targeting or protein stability. While transcription is the predominant level of gene regulation, research over the last decade has revealed that the abundance and distribution of a gene's final product also depends on posttranscriptional regulation, protein synthesis, posttranslational regulation, and protein stability (Gallie, 1998; Gutiérrez et al., 1999; Twyman, 2003; Twyman et al., 2002). Although alignment of Tc.C1, Tc.C2 and Tc.C3 revealed their very high identity with 81–86% identity of nucleic acid sequences and 78–85% identity of amino acid sequences, minor alterations of sequences in Tc.C1 and Tc.C2 may significantly affect expression and accumulation of the recombinant proteins via any step through the central dogma of transgene expression.

3.2. Anti-CarLA scFv–polyoleosin fusions bind onto the exsheathed L3 larvae

There are three main intraspecific epitopic groups of CarLA, each of the three scFv clones used here recognises a different group which means that together they should recognise more than 90% of the population of infective larvae (Maass et al., 2009). We tested the ability of a mixture of anti-CarLA scFv Tc.C1, Tc.C2, and Tc.C3 – polyoleosin fusions to bind the surface of L3 stage sheep nematode parasites by fluorescent staining. A strong signal was obtained using mixtures of either N- or C-terminal fusions of anti-CarLA scFv; no signal was detected with the negative controls (Fig. 3a). The comparative binding of individual and mixtures of anti-CarLA scFv–polyoleosin fusions was quantified by immuno-binding assays. Typically, binding efficacy was higher for the C-terminal scFv fusions and for both N- and C-terminal configurations the Tc.C2 clone had the highest degree of binding compared to Tc.C1 or Tc.C3 (Fig. 3b).

The difference in binding between the N- and C-terminal scFv fusions may be due to either a direct influence on the scFv freedom to move or indirectly by differences in the interactions between the amphipathic arms of oleosin (Tzen et al., 1992). Given that oleosin

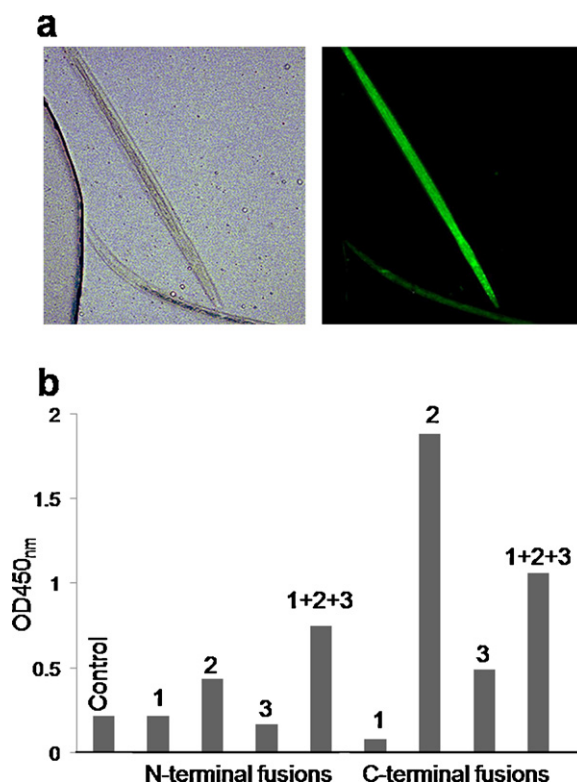


Fig. 3. (a) Immunofluorescent staining of living *T. colubriformis* L3 larvae reacted with Arabidopsis seed oil emulsion containing mixture of anti-CarLA scFv–polyoleosin fusions (Tc.C1+2+3). The stained larvae were visualised in bright-field microscopy (left), and green fluorescence (right) detected with rabbit anti-c-myc/FITC antibody. No signal was observed with the negative binding control using either $3 \times \text{myc}::3 \times \text{OLE}$ or $3 \times \text{OLE}::3 \times \text{myc}$. (b) Immuno-binding assay of exsheathed L3 larvae incubated with individual or mixtures of anti-CarLA scFv–polyoleosin fusions. Data are from one representative of two independent experiments with similar results. Either $3 \times \text{myc}::3 \times \text{OLE}$ or $3 \times \text{OLE}::3 \times \text{myc}$ were used as negative binding control. Both groups of N-terminal fusions (anti-CarLA Tc.C1::3 × OLE::3 × myc) and C-terminal fusions ($3 \times \text{myc}::3 \times \text{OLE}::\text{anti-CarLA Tc.C}$) were tested with the same prepared exsheathed larvae with equal quantities of scFv prepared at the same manner. 1, 2, and 3 stand for Tc.C1, Tc.C2, and Tc.C3, respectively. 1 + 2 + 3 (from either N- or C-terminal fusion) are the mixture of Tc.C1, Tc.C2 and Tc.C3 at equal amount.

targeting to OBs requires the appropriate location of the N-terminal hydrophilic arm (Abell et al., 1997), fusing a large recombinant protein to the N-terminus might interfere with the association of oleosin to the OB and therefore affect the stability of oleosin (or polyoleosin in this case) and function of fused recombinant proteins. Alternatively, fusion to the C-terminus may allow a greater portion of the scFvs to fold correctly. It should be noted that both Banilas et al. (2011) and Li et al. (2011) only reported on fusions to the C-terminus of oleosin.

The higher binding by the Tc.C2 clone may reflect a higher affinity or a larger proportion these epitopes (or access to them) on the larvae; similarly the opposite may explain the lack of binding activity by either N- or C-terminal Tc.C1 fusion. Alternatively, the lack of binding could be due to the relatively high ratio of OB emulsion:buffer required in order to compare an equal amount of Tc.C1 scFv with the other clones. Interestingly, the combination of N-terminal anti-CarLA Tc.C fusions resulted in enhanced biological activity compared to individual N-terminal scFv fusions, this was not true however of the combined C-terminal fusions which had a lower binding level than the individual anti-CarLA Tc.C2 C-terminal fusion (Fig. 3b). In this case it is likely that some of the reduced binding is simply coming from the dilution of the highest binding (C-terminal anti-CarLA Tc.C2 fusion) scFv.

3.3. Fusion to polyoleosin may provide a number of benefits

In comparison with the native 18-kDa *Arabidopsis* oleosin, Banilas et al. (2011) found that GFP accumulation was highest (2.3% of TSP) when it was fused between *Arabidopsis* oleosin (AtOleo) and maize oleosin (MzOleo) compared to MzOleo::GFP (0.18%), and AtOleo::MzOleo::GFP and $2 \times$ AtOleo::MzOleo::GFP (0.36% for both). Their results suggested that optimisation of the protein expression and stability may have to be determined empirically in terms of what fusion configuration was optimal. As such it would be interesting to investigate the influence of fusing of scFv between single units or between multimeric oleosins on protein yield.

In the last decade, engineering the monovalent structures of Fab, scFv and VHH in multivalent structures (multimeric conjugates) have been shown to increase functional affinity (Conrath et al., 2001; Winichayakul et al., 2009). Polyoleosin enables this to be achieved either through fusion of multiple clones between the oleosin repeats or by co-expressing different polyoleosin-scFv fusions, both would result in OBs decorated in multiple antibody fragments. The use of polyoleosin to create a multivalent macromolecule should not only increase functional sensitivity but also increase potency which would be particularly useful when faced with intraspecific variation of antigen epitopes, e.g., CarLA epitopes (Maass et al., 2009).

4. Conclusions

The successful expression of functional anti-CarLA scFv in *Arabidopsis* seeds as trimeric-polyoleosin fusions provides a novel use for polyoleosin to produce and deliver recombinant antibody fragments. OB-based formulations can be used as delivery agents in a variety of preparations including topical application as well as oral delivery. This flexibility lends itself to animal health especially where purity may be less of an issue. Oral intake of therapeutic agents (passive vaccines) in the form of feeding additives could provide direct benefits to the animal health and farming management while not invoking the immune system. However, as an oral drench it will need to be formulated to survive the passage through rumen and abomasum in order to be delivered intact into the animal digestive system. We anticipate exploring the production of polyoleosin fused scFv and VHH in established agricultural oil seed crops in order to provide sufficient material for testing in animal models.

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