

Single chain antibody fragments for ocular use produced at high levels in a commercial wheat variety

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Abstract

We are investigating the use of single chain antibody fragments (scFv) in eye drops for diagnosis and treatment of eye diseases. For ocular use, recombinant proteins must be free of bacterial endotoxin that causes inflammation in the eye. We required a means of generating high yields of scFvs with little endotoxin contamination. Using microprojectile bombardment we produced transgenic lines of the commercial wheat variety, Westonia, that express two scFvs that bind to CD4 or CD28 on the surface of rat thymocytes. A high level of expression of active scFv in the range 50–180 µg/g was measured by quantitative flow cytometry in crude extracts made from mature seeds. The levels of expression were stable over four generations of transgenic plants and mature seeds were stored for one year with little loss of scFv activity. Substantial purification of scFv was achieved by immobilised metal affinity chromatography. Compared to bacterial extracts, crude transgenic seed extracts contained only a small amount of endotoxin (150 EU/ml) that will be easily removed by purification. The transgenic wheat lines express functional scFv at levels comparable to production in bacteria and promise to be superior to bacteria for production of scFv pharmaceuticals for ocular use.

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1. Introduction

Monoclonal antibodies are important human therapeutic agents that are being administered as molecular traps or targeting devices to treat many human diseases including cancer, rheumatoid arthritis and some eye diseases (Maloney, 2003; Vogel et al., 2001; Keating and Perry, 2002; Rosenfeld et al., 2006). Whole antibodies, however, have disadvantages as therapeutic drugs for humans. They may elicit serious side effects, are immunogenic, and their large size restricts effective tissue penetration.

Genetic engineering permits the production of single chain antibody fragments (scFv) (Plückthun et al., 1996) that have fewer inflammatory properties than whole antibodies and exhibit better tissue penetration.

Our interest is in improving outcomes for patients with blinding eye disease. Whole antibody molecules penetrate poorly or not at all into the normal eye when administered systemically or applied topically (Thiel et al., 2002; Williams et al., 2005). Intra-ocular injection of antibodies has been tested and shown to produce a therapeutic effect (Heier et al., 2006), but long-term intra-ocular injection is not appropriate for many patients, especially those with early stage diseases. In such cases topical application is the preferred method for drug delivery to the eye. Therapeutic concentrations can often be achieved with few systemic side effects and many patients can manage self-administration of eye drops. We have discovered that purified scFv antibody fragments will penetrate into the eye when applied as an eye-drop, and do not cause intra-ocular inflammation

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(Thiel et al., 2002; Williams et al., 2005). Antibody fragments thus have substantial promise as topical drugs for ophthalmic use.

Antibody fragments are usually produced in bacterial culture (Plückthun et al., 1996); however, they must undergo extensive purification before formulation as eye drops. The most difficult part of the purification process is the removal of endotoxin derived from the bacterial cell wall. Even low amounts of endotoxin cause inflammation in the eye. The endotoxin load must be reduced over one million fold before the protein is deemed safe for ocular application, a process which is cumbersome and costly. Engineered antibodies and scFvs have been produced in eukaryote expression systems such as yeast, insect cell and mammalian cell culture (Ridder et al., 1995; zu Putlitz et al., 1990; Jost et al., 1994), which do not produce endotoxin. However, the yield of recombinant protein in mammalian systems, in particular, is extremely low (Verma et al., 1998) and there are limitations when scale-up is required, such that the cost may become prohibitive (Hood et al., 2002). In addition, proteins produced in mammalian cell culture may be contaminated with animal-based pathogens or viruses that must be removed before therapeutic use (Gomord et al., 2004).

Plants offer an alternate eukaryote expression system free of animal pathogens and with low endotoxin load. Tobacco was the first plant in which antibodies were expressed (Hiatt et al., 1989). They have since been expressed in a diverse range of plants. Seeds have evolved to facilitate the storage of proteins in a stable form. The developing seed contains molecular chaperones and disulphide isomerases to promote correct folding of antibodies, which, together with a low level of proteases and the desiccated nature of mature seeds, make the production of recombinant antibodies in seeds an advantageous option (Stöger et al., 2005). The cereal seeds of rice and barley are being investigated by biotechnology companies as potential platforms for molecular farming of proteins. Wheat has not received the same interest, possibly due to difficulties achieving reliable and efficient transformation (Janakiraman et al., 2002). A medically relevant scFv has been expressed in transgenic wheat on an experimental scale, and the level of expression was found to be considerably lower than that achieved for the same constructs in rice (Stöger et al., 2000). In many countries wheat is the major cereal crop with low production costs. It has potential as a commercial platform for recombinant antibody production if good expression levels of recombinant protein can be achieved. Here we describe the expression of two scFvs in a commercial high-yielding variety of wheat, and demonstrate that good expression of scFvs is obtained in transgenic wheat lines with low endotoxin contamination. The scFvs have specificity against the CD4 and CD28 molecules on the surface of rat thymocytes, the target cell used for detection of antibody binding herein. CD4 and CD28 are required for stimulation of some T lymphocytes during a cell-mediated immune response. We are exploring the potential of these scFvs to reduce the incidence of corneal graft rejection by blocking the immune response to the donor tissue that occurs in some recipients of corneal grafts.

2. Materials and methods

2.1. Single chain antibody constructs for expression in wheat

The construction of the anti CD4 and anti CD28 scFv genes and the production of the scFvs in *E. coli* have been reported previously (Thiel et al., 2002; Mavrangelos et al., 2001). Each gene was cloned into the vector pHB400, which incorporates a C-terminal polyhistidine tag (6His), useful for detection and later purification, and co-expresses the bacterial periplasmic chaperone Skp for improved yield of soluble scFv in bacteria (Mavrangelos et al., 2001). The anti CD4 and anti CD28 scFv genes were modified for expression in wheat by recombinant PCR. A 72 bp barley α -amylase signal sequence (nt 706–777 of clone HVAMY152, GenBank accession number X15226) was incorporated by the 5' primer along with a *Kpn* I restriction site. The 3' primer spanned the 6His tag region and incorporated a KDEL endoplasmic reticulum (ER) retention signal sequence (Schouten et al., 1996) and a *Kpn* I restriction site (Fig. 1). The PCR-modified scFv genes were digested with *Kpn* I and ligated into the vector pGBA2, between a maize ubiquitin promoter and intron (Christensen and Quail, 1996) and a maize zein terminator (Woo et al., 2001) producing pGBA2CD4 and pGBA2CD28. The pGBA2CD4 plasmid was digested with *Hae* II to produce a linear expression cassette containing the anti CD4 scFv (IGBA2CD4) (Fig. 1).

2.2. Selectable marker construct

The cyanamide hydratase gene (*cah*) (Weeks et al., 2000), was inserted into the pGBA2 vector between the ubiquitin promoter and zein terminator to form the selectable marker plasmid pGBA2Cah. The plasmid was digested with *Hae* II to produce a linear fragment containing the ubiquitin promoter, *cah* and maize zein terminator (IGBA2Cah). The *cah* gene confers resistance to cyanamide by converting cyanamide to urea.

2.3. Wheat transformation by microprojectile bombardment

Immature wheat seeds (cv Westonia) harvested from plants grown in a glasshouse were surface sterilised with multiple ethanol (70% v/v) and bleach (6.25% v/v) washes.

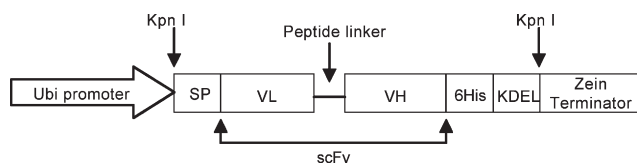


Fig. 1. The arrangement of the anti CD4/CD28 scFv expression cassette, modified for expression in wheat. Ubi, maize ubiquitin promoter with intron; SP, barley α -amylase ER-targeting signal peptide; VL, variable region immunoglobulin light chain; VH, variable region immunoglobulin heavy chain; 6His, polyhistidine tag; KDEL, ER retention signal; maize zein terminator; *Kpn* I restriction enzyme site.

The immature embryos were excised and placed on MS medium (Murashige and Skoog, 1962) containing 2.5 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) (C1 medium) for five to sixteen days prior to bombardment. Osmoticum treatments of target tissues, DNA precipitation and microprojectile bombardment were performed as described for sugarcane (Bower et al., 1996), with the exception that gold particles were used instead of tungsten particles as a carrier. Wheat tissues were bombarded with 150 µg of gold particles that had been coated with a mixture of 1 µg of pGBA2CD28 or 0.5 µg of IGBA2CD4 and 0.5 µg of IGBA2Cah.

2.4. Regeneration of transgenic wheat lines

Following bombardment the embryos were placed on C1 medium for two weeks at 24 °C and maintained in the dark. All subsequent subcultures were carried out at two-week intervals and maintained in the light. The callus was subcultured twice on C1 medium plus 40 mg/l cyanamide, once on MS medium containing 0.5 mg/l of 2,4-D plus 40 mg/l cyanamide, once on MS medium with 40 mg/l cyanamide and with no 2,4-D and then individual shoots were moved into tubes containing half strength MS medium plus 40 mg/l cyanamide. At three months the young plants were transplanted into pots in potting mix and grown in a glasshouse with a temperature cycle of 24 °C/12 °C (d/n). The fragile plants went through a hardening-off process over a two-week period.

2.5. PCR analysis of transgenic plants

DNA was extracted from leaf tissue of each transgenic T₀ plant using the Nucleon PhytoPure DNA Extraction Kit (GE Healthcare, Piscataway, NJ). The transgenic status of the regenerated plants was confirmed by PCR amplification of the anti CD4 or anti CD28 scFv genes from the leaf DNA. Seeds from all confirmed transgenic plants were planted to produce T₁ progeny. Further generations were grown from selected plants based on the scFv expression level and the phenotype of the plants.

2.6. Extraction of total soluble protein from wheat leaf or seeds

A piece of wheat leaf (~0.1 g) or ten seeds from a transgenic plant were weighed, ground to a fine power in liquid nitrogen using a pre-chilled mortar and pestle, and then homogenized in 1 ml of protein extraction buffer (50 M Tris–HCl, pH 7.8, 1 mM EDTA, 5% (w/v) polyvinylpolypyrrolidone (PVPP), and 1 mM phenylmethanesulfonyl fluoride (PMSF)). After centrifugation of the extract (30,000 × g, 4 °C, 20 min), the supernatant was stored at 4 °C. Sodium azide (20 mM) was added as a preservative. Extracts for purification were prepared from 10 g mature seeds and 40 ml extraction buffer (50 mM NaH₂PO₄ pH 8.0, 150 mM NaCl, 5% PVPP, 1 mM PMSF). Protein concentrations were determined using the Bradford assay (Bradford, 1976) using bovine serum albumin as the protein standard.

2.7. Western blotting

Mature seed protein extracts (containing 50 mg protein) were separated on 16% SDS-polyacrylamide gels and transferred to nitrocellulose membrane. ScFv protein was detected with anti polyHis antibody conjugated to horseradish peroxidase (1:10,000 dilution, CLONTECH, Mountain View, CA, USA) followed by enhanced chemiluminescence (ECL) Western blotting reagents (GE Healthcare, Piscataway, NJ, USA) and exposed to film.

2.8. Measurement of antibody fragment activity

The binding activity of anti CD4 or anti CD28 antibody fragments in wheat extracts was measured by high-sensitivity immunofluorescence on normal rat thymocytes and detected by flow cytometry as described previously (Thiel et al., 2002). The anti CD4 scFv protein standard was made in bacteria and purified by immobilised metal ion affinity chromatography and ion exchange chromatography as described previously (Brereton et al., 2005).

2.9. Endotoxin testing

Extracts were prepared to avoid the introduction of exogenous endotoxin. Equipment was cleaned with Etoclean (Sigma–Aldrich Inc., St. Louis, MO, USA) and solutions were prepared using sterile, pyrogen-free water (Baxter Healthcare Pty Ltd, Old Toongabbie, NSW, Australia). Endotoxin levels in extracts were measured by the Limulus Amebocyte lysate (LAL) test (Pyrotell, Associates of Cape Cod Inc, E Palmouth, MA, USA) according to the manufacturer's instructions.

2.10. Immobilised metal affinity chromatography

Crude extract from mature seeds was adjusted to 0.5 M NaCl, 20 mM imidazole, 0.5% sodium dodecyl sulphate (SDS), stirred at room temperature for 15 min and then centrifuged at 20,000 × g for 20 min at 4 °C. The supernatant was stirred with Profinity IMAC Ni-charged resin (Bio-Rad Laboratories, Hercules, CA) for 1 h at 4 °C to allow binding. The resin was recovered by centrifugation, washed by centrifugation with 50 mM NaH₂PO₄ pH 8.0, 20 mM imidazole, loaded into a column and eluted with the same buffer containing step-wise increases of imidazole to 30 mM, 120 mM and 250 mM.

3. Results

3.1. Production of transgenic wheat lines

Westonia wheat embryos were co-transformed by gold particle bombardment with plasmid pGBA2 containing either the anti CD4 or the anti CD28 scFv gene and pGBA2Cah (selectable marker), and putative transgenic wheat lines were selected by growth on cyanamide. Thirty-three putative transgenic plants were generated for the anti CD4 scFv, and 37 for the anti CD28

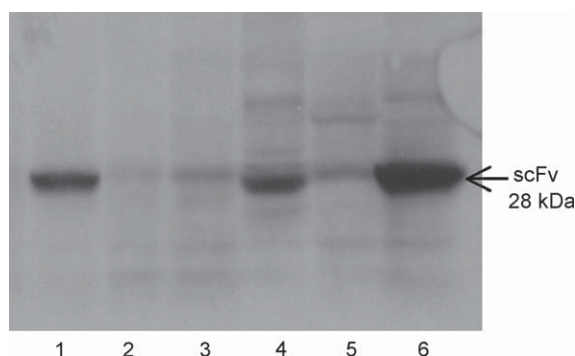


Fig. 2. Expression of scFv protein in crude extracts of mature seed from selected transgenic wheat lines by Western blot. ScFv protein was detected with anti poly-His antibody conjugated with horseradish peroxidase and visualised by ECL. Lanes 1, 2940.1.11 (CD4) positive; 2, non-transgenic Westonia (control); 3, 2912.2.3 (CD4) negative; 4, 3143.1 (CD28) positive; 5, 2767.1.36 (CD28) negative; 6, 3145.1 (CD28) positive. Detection of a weakly-stained band that ran just above the scFv bands is visible in lanes 1–5. This band is not regarded as anti CD4 or CD28 scFv, and is considered to be due to non-specific binding of the antibody to unrelated proteins of slightly different size.

scFv. Leaf tissue from each putative transgenic plant was tested by PCR amplification. Sixteen independent lines transgenic for the anti CD4 scFv gene and 16 independent lines transgenic for the anti CD28 scFv gene were obtained. The scFv gene from representative PCR-positive transgenic lines 2940.1 (anti CD4 scFv) and 3143.1 (anti CD28 scFv) was sequenced to confirm the identity. No mutations or frames shifts were found in either line.

Western blot analysis of soluble protein extracts of seed tissue from selected transgenic lines was used to detect expression of 6His-tagged scFv protein and to confirm that the protein produced was of the predicted size (Fig. 2). Four of six CD4 PCR-positive lines and three of four CD28 PCR-positive lines tested by Western blot showed detection of a 28 kDa scFv protein.

3.2. Detection of functional antibody fragments in crude wheat extracts

3.2.1. Anti CD4 scFv antibody fragments

Functional activity of the scFv was determined in parallel with protein detection in transgenic lines. For a small number of first generation plants (T_0), leaf extracts were incubated with rat thymocytes and the characteristic binding of scFv to CD4 on the surface of thymocytes was measured by flow cytometry. Binding activity was detected in three of six leaf extracts tested, however, the degree of binding was very low (mean fluorescence intensity (MFI) <10) in comparison to the binding of purified anti CD4 scFv made in bacteria (MFI 243) (Fig. 3A). Extracts made from mature seeds collected from the same T_0 plants showed considerably increased binding activity (MFI 50–100), but still less than purified bacterial scFv (Fig. 3B). Eleven of fourteen T_0 wheat lines transgenic for anti CD4 scFv showed binding activity in extracts of mature seeds. The four anti CD4 lines that showed expression of scFv protein by Western blot in the initial screen all showed binding activity.

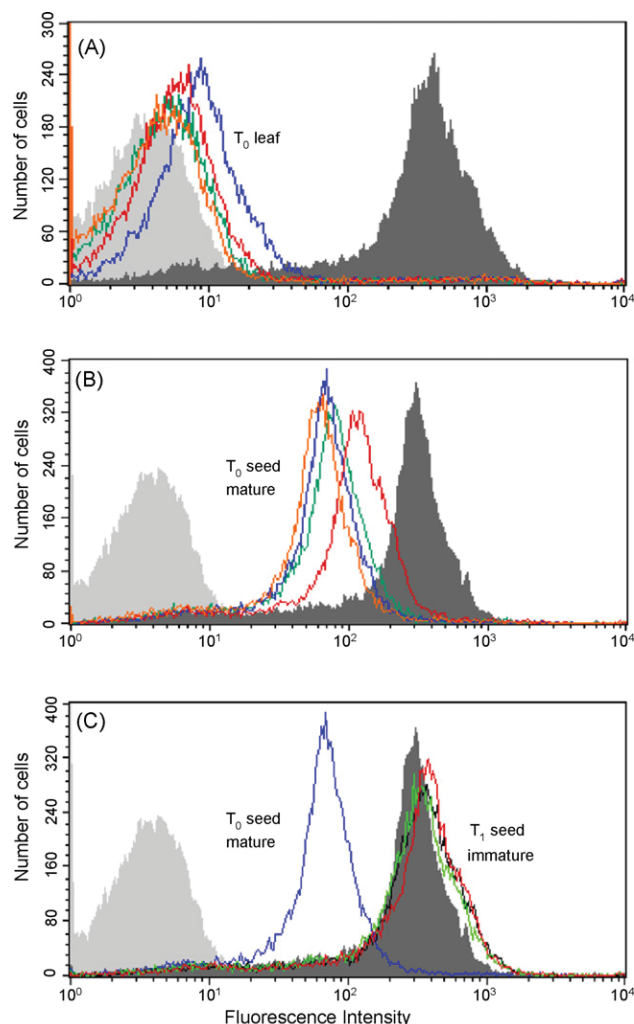


Fig. 3. Demonstration of binding of crude wheat extracts to CD4 on the surface of rat thymocytes by flow cytometry. In all panels: negative control, extract of non-transgenic Westonia wheat MFI 4.0 (light grey fill); positive control, purified anti CD4 scFv prepared in bacteria (2 μ g/ml) MFI 243 (A), 198 (B, C) (dark grey fill). MFI = mean fluorescence intensity. (A) Leaf extracts of four first generation T_0 wheat lines transgenic for anti CD4 scFv 2940.1 MFI 9.1 (blue), 2957.1 MFI 4.9 (green), 3012.2 MFI 5.8 (red), 3012.3 MFI 4.5 (orange). (B) Extracts prepared from mature seeds of the same four T_0 wheat lines, 2940.1 MFI 58 (blue), 2957.1 MFI 62 (green), 3012.2 MFI 94 (red), 3012.3 MFI 50 (orange). (C) Extracts prepared from mature seeds of 2940.1 T_0 wheat line MFI 58 (blue) and immature seeds from three representative progeny T_1 lines 2940.1.12 MFI 234 (green), 2940.1.14 MFI 268 (red), 2940.1.25 MFI 260 (black).

Propagation of seeds of selected lines to produce second generation (T_1) transgenic wheat lines revealed that the binding activity detected in seed extracts varied with the stage of maturity of the seed. Immature seeds produced extracts that showed a significantly greater degree of binding, similar to that seen with purified bacterial scFv (Fig. 3C). Seeds that had been allowed to ripen to maturity produced extracts that showed binding similar to mature seed extract from the parent T_0 plant (Fig. 4). This was observed consistently with T_1 , T_2 (Fig. 4) and T_3 (data not shown) generation plants. This was not due to the ability to extract more soluble protein from the softer immature seed. The amount of soluble protein extracted from mature seed (11.4 ± 2.8 mg/ml) was greater than that from immature

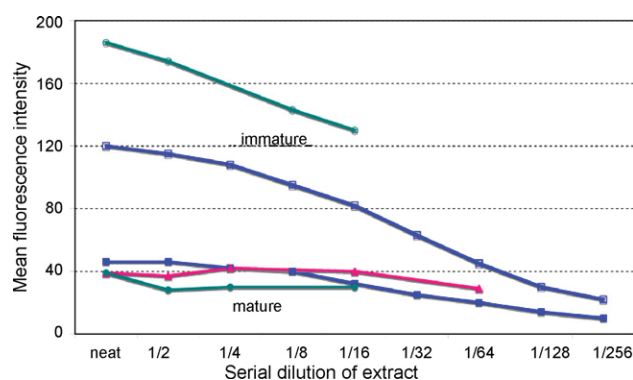


Fig. 4. CD4 binding activity in seed extracts from different generations of wheat plants and at different stages of seed maturity. Binding to rat thymocytes was determined by flow cytometry and is expressed as the variation in mean fluorescence intensity of the thymocyte population following serial dilution of seed extracts. Seed from plants: T₀ 2940.1 mature (solid triangle), T₁ 2940.1.25 immature (open circle), T₁ 2940.1.25 mature (solid circle), T₂ 2940.1.25.28 immature (open square), T₂ 2940.1.25.28 mature (solid square).

seed (8.7 ± 1.5 mg/ml). Serial dilution of extracts from immature seeds decreased the binding activity whereas it was possible to dilute extracts from mature seeds serially several times before the binding activity decreased appreciably (Fig. 4). This suggested that the mature seed extracts contained a binding inhibitor not present in immature seed extracts, rather than a lower amount of functional scFv.

3.2.2. Anti CD28 scFv antibody fragments

Functional activity of anti CD28 scFv was also tested by incubation with rat thymocytes, and detected by flow cytometry. The assay is less sensitive for the detection of binding to CD28 than to CD4 as the amount of CD28 expressed on the surface of thymocytes is lower than CD4 expression. Binding activity was not detected in leaf extracts of first generation (T₀) plants transgenic for anti CD28 scFv but was readily detected in immature and mature seed extracts from four of the six T₀ plants tested. The binding activity of mature seed extract was lower (MFI 10) than that of immature seed extract (MFI 21), which compared favourably to the binding of purified anti CD28 scFv produced in bacteria (MFI 16) (Fig. 5A). A decrease in binding activity with seed maturation was also seen in subsequent generations of wheat lines expressing anti CD28 scFv (Fig. 5B). Of three anti CD28 lines that showed expression of scFv protein by Western blot, only two showed binding activity.

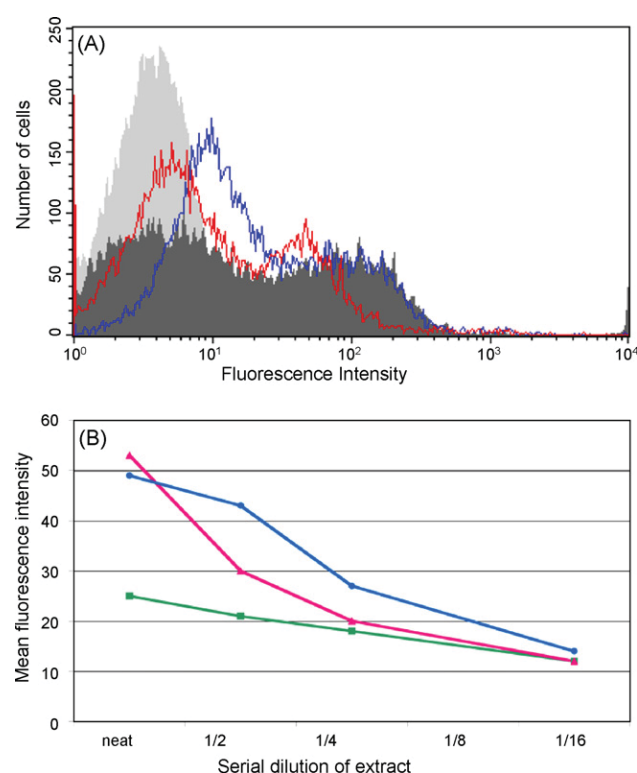


Fig. 5. Demonstration of binding of crude wheat extracts to CD28 on the surface of rat thymocytes by flow cytometry. (A) Flow cytometry histograms showing binding of crude seed extracts from two T₀ wheat lines transgenic for anti CD28 scFv: immature 3143.1 seed MFI 21 (blue); mature 3145.1 seed MFI 10 (red); negative control, seed extract of non-transgenic Westonia wheat MFI 4 (light grey fill); positive control, purified anti CD28 scFv prepared in bacteria MFI 16 (dark grey fill). (B) Titration of binding activity in crude extracts from: stored mature seed from T₀ plant 3145.1 (green square); immature seed of two progeny T₁ plants 3145.1.1 (pink triangle), 3145.1.13 (blue circle).

3.3. Stability of scFv activity

Mature seeds were stored for up to one year at 4 °C prior to extract preparation and assay by flow cytometry. Titration of the extracts showed that binding activity in the seeds was appreciably preserved over this time (Table 1).

3.4. Yield of scFv in crude seed extract

An estimation of the amount of functionally active scFv protein present in crude wheat extract was made by flow cytometry,

Table 1
Effect of storage of mature seeds on binding activity of anti CD4 scFv

Wheat line	Seed storage time	Protein concentration (mg/ml)	Normalised mean fluorescence intensity ^a following dilution of crude seed extract			
			1/1	1/2	1/4	1/16
2940.1.25	1 day	11	62	62	28	26
2940.1.25	4 months	12	39	28	30	30
2940.1.25.28	1 day	11	47	50	36	39
2940.1.25.28	1 year	10	39	35	23	21

^a Binding of extracts to rat thymocytes was determined by flow cytometry. Binding determined on different preparations of thymocytes was normalised relative to the binding of a standard preparation of purified anti CD4 scFv made in bacteria.

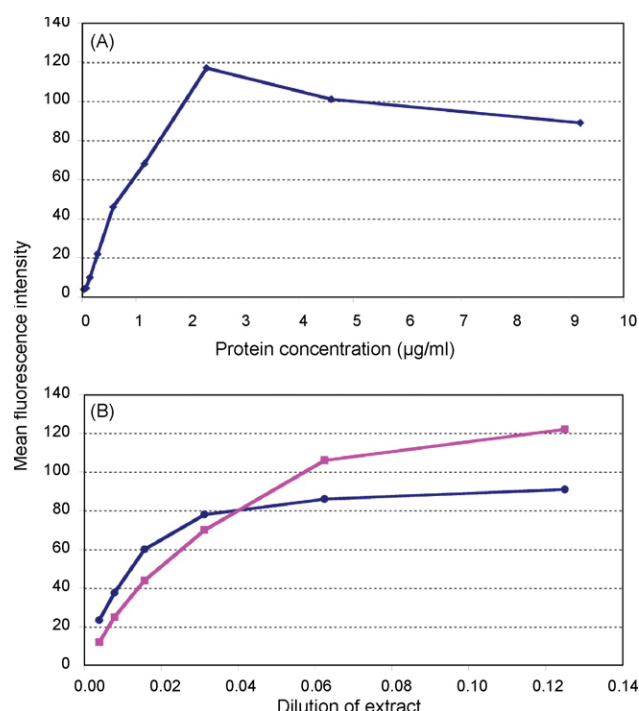


Fig. 6. Estimation of the amount of active scFv protein in crude seed extracts by flow cytometry on rat thymocytes. (A) Standard curve prepared by titration of pure anti CD4 scFv. (B) Titration of crude extracts of anti CD4 scFv prepared from mature seeds of T₃ wheat line 2940.1.25.10.13 (blue circle) and bacteria (pink square). Titrations were performed concurrently on the same preparation of thymocytes.

by comparing MFI values of a titration series of the crude extract with a titration series of highly purified bacterial anti CD4 scFv of known concentration, both titrations performed at the same time on the same preparation of thymocytes. Calculations were made using MFI values falling within the linear portions of both titration curves where the MFI is proportional to scFv concentration (Fig. 6). Crude extract from mature seeds of the T₃ wheat line 2940.1.25.10.13 was estimated to contain 65 µg functionally active anti CD4 scFv/ml. Typically, small scale seed extracts contained 20–65 µg scFv/ml, which reflects 50–180 µg scFv/g seed. This compares favourably with crude bacterial extract that contained 40 µg functionally active anti CD4 scFv/ml (Fig. 6), equivalent to 100 µg/g wet weight of *E. coli*.

3.5. Purification of anti CD4 scFv by immobilised metal affinity chromatography

Prior to chromatography 0.5% SDS was added to the crude extract to decrease the non-specific binding of proteins to the IMAC resin. This step also removed some of the contaminating proteins by precipitation. Substantial purification of anti CD4 scFv was achieved by affinity chromatography on IMAC resin (Fig. 7A). Most of the active scFv was eluted in the fraction between 30 mM and 120 mM imidazole. The extent of binding of the partially purified scFv to thymocytes (maximum mean fluorescence intensity observed in flow cytometry) was significantly greater than for crude extract of mature seeds (Fig. 7B), suggesting a binding inhibitor was removed during purification.

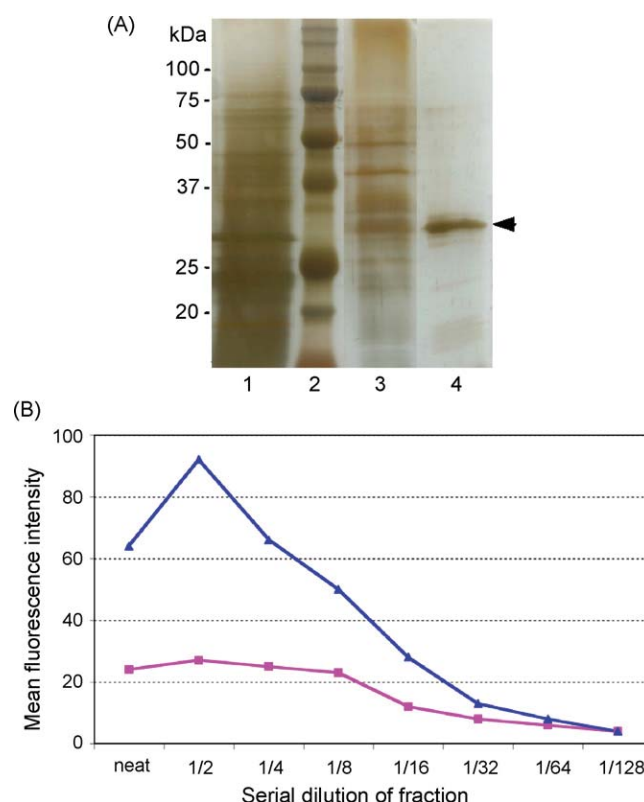


Fig. 7. Partial purification of anti CD4 scFv from mature seed extract by immobilised metal affinity chromatography (IMAC). (A) Silver-stained polyacrylamide gel. Lanes 1, crude seed extract (2.5 µg); 2, protein size standards; 3, IMAC fraction eluted between 30 mM and 120 mM imidazole; 4, purified anti CD4 scFv made in bacteria (0.5 µg). The position of the scFv band is indicated by arrow. (B) Titration of CD4 binding activity in the scFv fraction eluted from IMAC resin was determined by flow cytometry on rat thymocytes and was compared with binding of the crude extract. Crude extract from mature seeds (pink square); IMAC fraction eluted between 30 mM and 120 mM imidazole (blue triangle).

3.6. Endotoxin contamination in wheat extracts

Crude extract prepared from stored mature seeds of anti CD4 wheat line 2940.1.25.10 (T₂) was found to contain approximately 150 endotoxin units (EU)/ml. Crude extracts of *E. coli* expressing anti CD4 scFv typically contain more than 10⁶ EU/ml. Following IMAC chromatography the fractions with anti CD4 activity contained approximately 30 EU/ml.

3.7. Productivity of transgenic lines

The transgenic wheat lines had differing plant heights and yields. An assessment of which lines to continue to characterise was made at the T₁ generation on the basis of the binding activity of extracts made from mature seeds and the phenotype characteristics being similar to the non-transformed Westonia variety. The plant seed weight at the T₁ generation from the anti CD4 line 2940.1 was 6.3 g, similar to the seed yield from non-transformed Westonia plants grown in the glasshouse (6.3–7.9 g). The anti CD28 line 3145.1 yielded 12.1 g seed at T₁, well above that of Westonia plants. This pattern was consistent over subsequent generations of these transgenic lines (data not shown).

4. Discussion

Recombinant scFv proteins for therapeutic or diagnostic use in the eye must be essentially free of endotoxin to avoid inflammatory side effects. ScFv proteins produced in bacterial culture require extensive purification to remove large amounts of contaminating endotoxin, a process that leads to significantly reduced yield and increased production costs. Here we report the generation of transgenic wheat lines that express high levels of functionally active scFvs with only minor endotoxin contamination that will be easily removed during purification.

Two existing scFv genes were used in this study. One encodes an anti CD4 scFv that has been purified routinely from bacterial cultures and used extensively in corneal penetration studies (Thiel et al., 2002; Williams et al., 2005; Brereton et al., 2005). The other encodes an anti CD28 scFv that has also been produced previously in *E. coli* (Mavrangelos et al., 2001). The scFv genes were modified for expression in wheat by the insertion of sequences encoding a barley α -amylase ER-targeting signal peptide and a KDEL ER retention signal, to direct translation to the ER and promote and maintain correct scFv folding. The scFv genes were expressed under the control of the maize ubiquitin promoter which has been shown previously to produce transgene expression in most wheat tissues with highest levels in the endosperm (Stöger et al., 1999).

Very low or no binding activity was detected in leaf extracts of first generation (T_0) plants whereas seed extracts from the same plants showed significantly higher binding activity. A similar trend was seen with the ubiquitin promoter in *gusA* transgenic wheat (Stöger et al., 1999). Two wheat lines expressing anti CD28 scFv have been propagated for three generations and one wheat line expressing anti CD4 scFv has been propagated for four generations with consistently high levels of expression of functional scFvs in seeds. The yield of functional anti CD4 scFv in crude extract of one, fourth generation (T_3), line was estimated at 180 $\mu\text{g/g}$ mature seed, however, there was variation between sibling lines with yields in the range 50–180 $\mu\text{g/g}$ mature seed. Bacterial production is generally considered to provide the highest yield of scFv protein (Verma et al., 1998). We have generated wheat lines that produce amounts of active scFv in crude extracts of mature seeds that compare very favourably with our usual yield in crude bacterial extracts (100 $\mu\text{g/g}$ wet weight of *E. coli*).

The endotoxin load in crude seed extracts was at least 5000 times lower than in bacterial extracts. Care was taken to avoid introducing endotoxin during the extraction process so it is likely the residual endotoxin was derived from gram-negative bacteria resident on the wheat seed during growth and storage. It is expected that small amounts of residual endotoxin will be removed easily during purification without dedicated removal protocols that are costly and invariably incur protein losses. This would clearly be an advantage for production of scFv-based ocular pharmaceuticals.

Mature seeds have been stored for up to a year with little reduction in the amount of functional scFv that could be recovered in crude extracts. This has clear advantages for potential commercial production of scFv-derived pharmaceuticals in

wheat seeds, where low storage and transport costs should aid in decreasing the production cost.

The variability observed in plant height and seed yield of the scFv transgenic lines is consistent with somaclonal variation in wheat (Ivanov et al., 1998). At least one of the anti CD4 and anti CD28 scFv transgenic lines gave a seed yield comparable or above that of Westonia, which is a commercial wheat variety classed as Australian Premium White. In Western Australia, Westonia yields 2.6 t/ha on average. The predicted yield of crude scFv from mature seeds of the anti CD4 scFv lines is estimated to be in the range 130–450 g/ha.

It is noteworthy that we found across several generations that immature seeds consistently produced crude extracts that bound to thymocytes to a greater extent than did crude extracts of mature seeds. This phenomenon did not reflect the concentration of functional scFv in the respective seed extracts. It has been reported previously that the ubiquitin promoter was active throughout seed development with GUS activity maintained in mature seeds of *gusA* transgenic wheat (Stöger et al., 1999). The lower maximum binding of mature seed extracts was possibly caused by the presence of a binding competitor or inhibitor in the extracts that limited the scFv binding to thymocytes. Partial purification of mature seed extract was achieved by immobilised metal affinity chromatography and resulted in a significantly greater extent of binding to CD4 on thymocytes, similar to that of immature seed extracts and purified bacterial scFv. The type and ratio of seed proteins are known to change during seed development and maturation (Bénétrix et al., 1994). It is possible that one or more of the late accumulating or modified proteins in crude extracts of mature seeds had an inhibitory effect on the binding of scFv protein to thymocytes.

In conclusion, this work demonstrates the potential of producing recombinant antibody fragments for ocular use in a high-yielding commercial wheat variety.

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