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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.

Keywords: Antibody fragments; Ocular therapy; Eye drops; Plant-made pharmaceutical; Wheat transformation; Molecular farming

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 (lGBA2CD4) (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 (lGBA2Cah). 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.

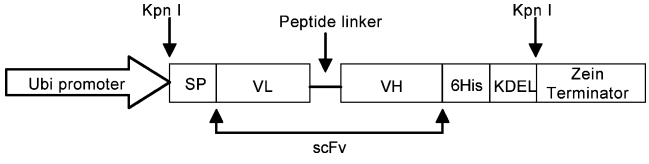

Fig. 1. The arrangement of the anti CD4/CD28 scFv expression cassette, modified for expression in wheat. Ubi, maize ubiquitin promoter with intron; SP, barley α-amylase ER-targeting signal peptide; VL, variable region immunoglobulin light chain; VH, variable region immunoglobulin heavy chain; 6His, polyhistidine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