Production of a functional single chain antibody attached to the surface of a plant virus

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Abstract A potato virus X (PVX) vector was used to express a single chain antibody fragment (scFv) against the herbicide diuron, as a fusion to the viral coat protein. The modified virus accumulated in inoculated *Nicotiana clevelandii* plants and assembled to give virus particles carrying the antibody fragment. Electron microscopy was used to show that virus particles from infected leaf sap were specifically trapped on grids coated with a diuron-BSA conjugate. The results demonstrate that the PVX vector can be used as a presentation system for functional scFv.

Key words: Single chain antibody fragment; Virus particle; Potato virus X

1. Introduction

Plant virus vectors are being used increasingly for the expression of foreign genes in plants, and a number of different viruses have been successfully developed into tools for gene delivery [1]. Due to the high level of accumulation of plant viruses, and the correspondingly high levels of viral gene expression, much interest has focused on developing virus vectors for the production of valuable proteins in plants. Several gene insertion vectors have been developed to allow the production of foreign proteins, either free in the plant cytoplasm, or targeted to cellular compartments [2–5].

An alternative to free protein production is to express foreign peptides and proteins as fusions to the viral coat protein, which, following virus assembly, can display the foreign sequence at the surface of the assembled particle. Research in this area has focused on the development of plant viruses carrying antigenic epitopes from human or animal pathogens with the aim of developing novel recombinant vaccines. Cowpea mosaic virus (CPMV) forms icosahedral particles, and is one of the best-studied systems for peptide presentation on the surface of a plant virus [6]. The development of CPMV as a peptide presentation system mirrored previous studies using the structurally related poliovirus [7]. CPMV has been used to present a number of different antigenic peptides on the particle surface [8,9]. Similar antigen presentation systems have been described for the icosahedral tomato bushy stunt virus [10], the bacilliform alfalfa mosaic virus (AlMV) [11], and the rod-shaped tobacco mosaic virus (TMV) [12–14]. The value of this approach is demonstrated by recent reports of protective immunity against rabies and foot-and-mouth disease virus (FMDV), conferred respectively by recombinant AlMV and CPMV particles carrying antigenic epitopes from the corresponding pathogen [15,16]. However, despite successes in presentation of antigenic peptides on the surface of TMV, CPMV and AlMV particles, the size of peptide fusions that still permit virus assembly is limited. Only peptides of less than 22 amino acids have been described for TMV-coat protein (CP) fusions, whereas for recombinant CPMV and AlMV particles, fusions have been limited to 30 and 47 amino acids, respectively [11,17].

We previously described a potato virus X (PVX)-based vector that allowed the production of virus particles decorated at their outer surface with the 27 kDa (237 amino acid) green fluorescent protein (GFP) as a fusion to the viral CP [18]. Although the GFP-CP fusion protein was unable on its own to assemble into virus, it was found that assembly of particles occurred when free CP was present in addition to the GFP-CP fusion. The requirement for free CP, in addition to the fusion protein, presumably reflects steric constraints on particle assembly imposed by the addition of foreign sequence. To provide both GFP-CP fusion protein and free CP, a strategy was developed that used the 16 amino acid 2A peptide from FMDV, positioned as a translational fusion between the amino-terminal GFP and the carboxy-terminal CP domains. The 2A peptide, which mediates a co-translational processing event in the maturation of the FMDV polyprotein, is also able to process synthetic polypeptides [19,20]. In plants infected by the modified PVX, accumulation of cleaved CP and GFP-2A along with uncleaved GFP-2A-CP fusion protein was observed. The GFP-2A-CP fusion protein subunits, in combination with free CP subunits, could assemble into filamentous rod-shaped particles that were decorated at their outer radius with GFP. Furthermore, the modified virus retained the ability to move locally and systemically through the plant [18].

The utility of a GFP-tagged virus in studying viral pathogenesis is well established [18,21]; however, the possibility of tagging other proteins to PVX particles provides the opportunity for a wide range of biotechnological applications. In order to demonstrate the production of a functional recombinant protein attached to PVX CP we investigated the expression and presentation of a single chain antobody fragment (scFv) against the herbicide diuron in plants.

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Abbreviations: PVX, potato virus X; CP, coat protein; scFv, single chain antibody fragment; CPMV, cowpea mosaic virus; AlMV, alfalfa mosaic virus; TMV, tobacco mosaic virus; GFP, green fluorescent protein; FMDV, foot-and-mouth disease virus; PCR, polymerase chain reaction

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2. Materials and methods

2.1. Plasmid construction

Standard DNA manipulation techniques were used throughout [22]. Plasmid L41 (L. Smolenska and S. Santa Cruz, unpublished) is based on pTXS.GFP-CP [18] but replaces the sequence encoding the two N-terminal amino acids of the FMDV 2A linker peptide with the recognition sequences for the restriction enzymes *Bsp*EI and *Xba*I. L41 was digested with *Eag*I and *Bsp*EI to delete the GFP coding sequence. The plasmid pSDLG2-FLAG, which encodes the anti-diuron scFv fragment [23], was used as template for PCR amplification with oligonucleotide primers designed to introduce recognition sequences for *Eag*I and *Bsp*EI at the 5′ and 3′ ends, respectively, of the amplified DNA. Following digestion with *Eag*I and *Bsp*EI the PCR product encoding the scFv was ligated to L41, replacing the deleted GFP sequence. The resulting plasmid, pTXS.αD-CP, encodes a translational fusion between the scFv, the 2A peptide and the CP. pTXS.αD-CP was digested with the restriction enzymes *Cla*I and *Eag*I to allow the insertion of an epitope tag at the amino-terminus of the polyprotein coding sequence. Two complementary oligonucleotides, encoding the eight amino acid FLAG tag, were annealed and ligated to pTXS.αD-CP to give pTXS.FαD-CP.

2.2. In vitro transcription and plant inoculation

In vitro run-off transcripts were synthesised from *Spe*I-digested pTXS.FαD-CP using a T7 RNA polymerase transcription kit (Ambion, Austin, TX, USA). Transcripts were inoculated directly to young leaves of *Nicotiana clevelandii* plants by rubbing aluminium oxide-dusted leaves. Each leaf was inoculated with transcript products derived from 0.4 μg of plasmid template.

2.3. Immunoblot analysis

Protein extracts from inoculated leaf tissue, harvested 5 days post inoculation (dpi), were prepared for SDS-polyacrylamide gel electrophoresis and blotting to nitrocellulose as described previously [18]. The M5 monoclonal antibody raised against the FLAG epitope (Sigma, St Louis, MO, USA) and rabbit polyclonal antiserum raised against PVX coat protein were used for immunodetection.

2.4. Immunosorbent electron microscopy

Electron microscope grids with a carbon support film were coated with diuron-BSA conjugate at a concentration of 0.01 μg/ml and control grids were coated with BSA alone at the same concentration. Coated grids were incubated on drops of sap from virus-infected leaf tissue, and then negatively stained with 2% sodium phosphotungstate (pH7) before observation using a JEOL 1200 electron microscope (JEOL, London, UK). All immunosorbent electron microscopy methods used have been described previously [24]. Diuron-BSA conjugate was prepared using a standard protocol for the conjugation of carboxylic acid haptens to proteins [25].

3. Results and discussion

3.1. Plant inoculation and protein analysis

In vitro run-off transcripts synthesised from pTXS.FαD-CP (Fig. 1) were infectious when inoculated to plants. Virus derived from transcript inoculation is subsequently referred to as PVX.FαD-CP. Symptoms of viral infection were observed by 5 dpi on inoculated leaves, and by 8 dpi on non-inoculated leaves (data not shown). Western blot analysis of protein extracts from inoculated *N. clevelandii* leaves, harvested at 4 dpi,

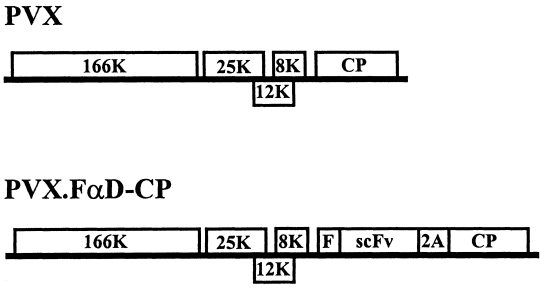

Fig. 1. Organisation of the wild-type PVX and PVX.FαD-CP genomes. Schematic representation (not to scale) of the wild-type PVX and recombinant PVX.FαD-CP viral genomes. Lines represent untranslated sequence and boxes represent coding sequences. The size of virus-coded proteins is shown (kDa); CP, coat protein; F, FLAG tag; scFv, single chain antibody.

demonstrated the presence of both CP and FLAG antigen. Immunoblots probed with antiserum raised against the CP (Fig. 2A) show two immunoreactive bands, corresponding to the scFv-2A-CP fusion protein and the cleaved CP. Similarly, blots probed with antiserum raised against the FLAG epitope also show two bands, corresponding to the unprocessed fusion protein and to cleaved scFv-2A (Fig. 2B). Thus, translation of the synthetic scFv-2A-CP polyprotein results in the accumulation of both unprocessed polyprotein and the products of 2A-mediated processing, scFv-2A and CP.

Based on analysis of immunoblots probed with antibody raised against the CP, and comparison with purified CP standards, we estimate that in PVX.FαD-CP infected leaf tissue, CP accumulated to approximately 200–500 μg/g fresh weight tissue by 4 dpi (data not shown). In the absence of a standard we were unable to quantify directly the level of scFv produced, however, CP and scFv are produced at exactly the same level, and approximately 50% of detected scFv antigen is present as fusion to CP (Fig. 2). We therefore estimate the level of scFv-2A-CP fusion protein as approximately 100–250 μg/g fresh weight tissue by 4 dpi.

3.2. Immunotrapping of virus particles

To establish whether virus particles were assembled that contained the scFv-2A-CP polyprotein, and whether the scFv domain remained functional for antigen binding, sap from PVX.FαD-CP-infected leaves was incubated with carbon support film grids carrying either a diuron-BSA conjugate or BSA alone. The results of this immunosorbent antigen binding assay (Table 1) show a 60-fold increase in virus particles trapped on diuron-BSA-coated films compared with BSA-coated control films. In contrast, similar numbers of particles were trapped on both diuron-BSA and BSA films incubated with sap prepared from leaf tissue infected with wild-type PVX, confirming that the trapping of PVX.FαD-

Table 1
Immunotrapping of virus particles on coated carbon support film electron microscopy grids

Inoculum	Grid coating	Virus particles/1000 μm^2	Factorial increase[a]
PVX.FαD-CP	diuron-BSA	1490	62.1
	BSA	24	–
Wild-type PVX	diuron-BSA	111	1.3
	BSA	85	–

[a]Relative increase in particle trapping on diuron-BSA-coated grids compared to grids coated with BSA alone.

CP particles on diuron-BSA-coated grids was due to incorporation of the scFv-2A-CP polyprotein into virus particles. This specific trapping of PVX.FαD-CP virus particles, on grids coated with the diuron-BSA conjugate, provides clear evidence that functional scFv could be incorporated into virus particles as a fusion to the viral CP. The PVX.FαD-CP particles trapped on diuron-BSA-coated grids appeared as filamentous rods typical of wild-type PVX particles (Fig. 3).

The antigen binding properties of antibodies are exploited in many biological, biotechnological and medical applications and plants are increasingly being used as expression systems for antibody production [26]. Expression of both complete [27,28] and engineered antibodies [29,30] in transgenic plants is well documented. Furthermore, the functionality of transgenically expressed antibodies has been demonstrated both in situ [29,30] and ex situ [31]. However, all plant-based antibody expression systems described so far have relied on the preparation of transgenic plant lines. Here we describe an alternative system for rapidly producing functional scFv in plants.

These data extend previous results and demonstrate that the PVX vector can be used for the presentation of functional scFv antibody fragments in plants. In the case of the scFv used in this study, which recognises the herbicide diuron, potential in situ applications include remediation of contaminated soils and waterways. The practical benefits of using plants as cheap, self-maintaining, bioreactors for the production of antibodies has been exemplified recently by the demonstration of effective preventative immunotherapy using a secretory antibody produced in transgenic plants [31]. The development of viruses as expression tools for the production of functional scFv will extend the utility of plant-based antibody production and provides a novel means for directing high-level expression of a carrier-linked scFv in plants.

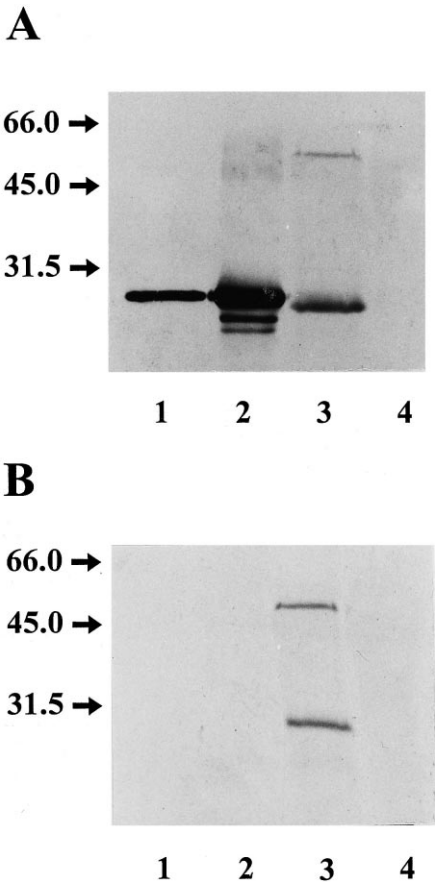

Fig. 2. Immunoblot analysis of total leaf protein from *N. clevelandii* leaves, probed with either anti-CP antiserum (A) or anti-M5 Flag monoclonal antibody (B). Lane 1 contained 100 ng of PVX CP. Lane 2 contained protein from wild-type PVX-infected leaves, lanes 3 and 4 contained protein from plants inoculated with in vitro transcripts synthesised from pTXS.FαD-CP or mock-inoculated control plants respectively. The M_r of the native PVX CP is 25.1 kDa, the M_r of the scFv-2A-CP fusion protein is 55.5 kDa and the predicted M_r values of scFv-2A and CP released following 2A-mediated processing are 30.7 kDa and 24.8 kDa, respectively. The M_r values of standards ($\times 10^{-3}$) are shown on the left.

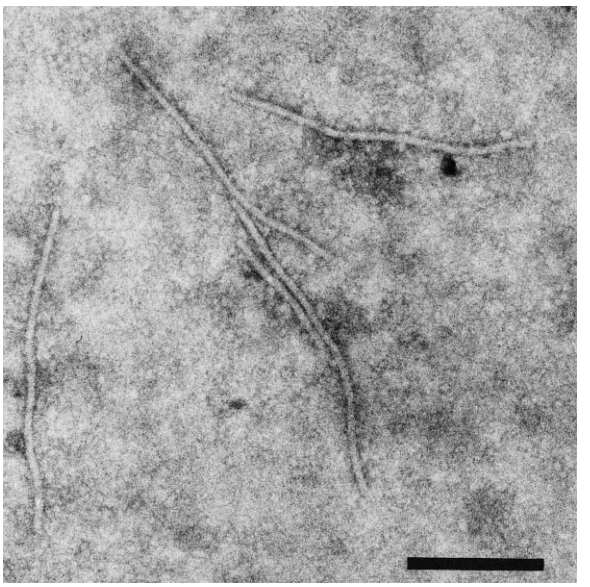

Fig. 3. Electron micrograph showing PVX.FαD-CP particles trapped on diuron-BSA-coated carbon support film grids (bar = 200 nm).

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