Use of phage display for isolation and characterization of single-chain variable fragments against dihydroflavonol 4-reductase from Petunia hybrida

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Abstract To isolate specific single-chain variable (scFv) fragments against dihydroflavonol 4-reductase (DFR) from Petunia hybrida the phage display technology was used. DFR was overproduced in Escherichia coli, purified and used for immunization. From DFR-immunized mice, a phage display library was made starting from spleen mRNA using an optimized set of primers for VH and VL amplification. Several rounds of panning against recombinant DFR yielded five different scFv fragments, confirmed by subsequent DNA sequencing. They all specifically bound to recombinant DFR in ELISA and DFR in flower extracts on Western blot. These results show that phage display is a promising technology in plant molecular biology to obtain specific recombinant antibodies not only for ELISA and Western blot but also for in vivo applications in the long run.

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Key words: Dihydroflavonol 4-reductase; Immunomodulation; Phage display; Recombinant antibody; Single-chain variable fragment; Petunia hybrida

1. Introduction

Since Hiatt and co-workers have shown that plants can express antibodies [1], several research groups have started to make use of the high specificity of antibody-antigen interaction for applications such as virus resistance [2,3], interference with light reception [4], or modulation of phytohormone action [5]. Each of these applications is based on a similar principle, namely to modulate the activity of the target molecule by interaction with a specific immunoglobulin in an appropriate plant cell compartment. The two main bottlenecks for immunomodulation-based applications in heterologous systems are the generation of antibodies with high specificity and affinity, and the isolation of the structural genes encoding these. Although hybridoma technology enables production of highly specific monoclonal antibodies, their generation is labor intensive and requires the use of animals, animal tissue cell culture and expensive equipment. Many plant molecular biology laboratories do not have these facilities nor the experience to generate monoclonal antibodies. Moreover, the hybridoma technology does not allow immediate isolation and cloning of immunoglobulin-encoding genes. However, the phage display technology offers a new way of producing antibodies mainly using basic recombinant DNA techniques, which allows coselection of recombinant antibodies and their respective genes in any molecular biology laboratory [6,7].

To evaluate the efficiency to generate scFv fragments against a plant enzyme by phage display technology, we used dihydroflavonol 4-reductase (DFR) from Petunia hybrida as a target. DFR is a key enzyme in the flavonoid biosynthetic pathway, responsible for color development in flowers [8]. It has previously been shown that knockout of the dfr gene causes loss of flower color [8,9]. Hence, this enzyme may be a good model system to investigate the efficiency and stability of enzyme inhibition in planta by recombinant antibody expression. Here, we describe the isolation and characterization of DFR-specific scFv fragments against DFR using phage display.

2. Materials and methods

2.1. Overexpression of DFR in E. coli

The full-length cDNA of DFR [8] was amplified by PCR using two primers (Table 1A) that allowed the incorporation of a BamHI and a BglII restriction site at the 5′ and 3′ end, respectively. The PCR fragment was cloned in a Smal-linearized pGEM2 cloning vector and confirmed by sequencing. The BamHI/BglII cDNA fragment was cloned in-frame into the BamHI-linearized expression vector pET3a [11] using the E. coli DH5α [12]. Plasmids from clones with correct inserts were transformed in the E. coli expressor strain BL21(D3) for expression [11]. Recombinant clones were grown overnight at 37°C in liquid LB medium containing 100 μg/ml carbenicillin, diluted 100-fold in the same medium and grown until an OD600 of 0.8. Recombinant DFR (DFR™) expression was induced by adding 1 mM isopropyl β-D-thiogalactopyranoside and analyzed by SDS-PAGE.

2.2. Purification of DFR™ from E. coli extracts

A DFR™-producing BL21(D3) clone was scaled up to one liter. Six hours after induction, the bacteria were pelleted (8000×g/10 min/4°C). The cells were washed in TE buffer, dissolved in Mcllvain C buffer [13], containing 1 mM phenylmethylsulfonyl fluoride, and so-

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Abbreviations: BSA, bovine serum albumin; cfu, colony-forming units; C51, constant region of heavy chain; Cl, constant region of light chain; DFR, dihydroflavonol 4-reductase; DFR™, recombinant DFR; ELISA, enzyme-linked immunosorbent assay; FUE, fluorescence units; Ig, immunoglobulin; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; scFv, single-chain variable fragment; SDS, sodium dodecyl sulfate; SOE, splice-overlap extension; VH, variable domain; VH, variable region of heavy chain; VH, variable region of light chain; VH, variable region of k light chain; VH, variable region of λ light chain; VH, variable region of IgG, heavy chain; VH, variable region of IgG, heavy chain

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2.5. Enrichment and screening of the phage display library

Screening was done by phage-ELISA. Monoclonal phage stocks were prepared in microtiter plates according to the Recombinant Phage Antibody System protocol (RPAS; Pharmacia). For each phage clone, one well was coated with 5 μg/ml DFRrec and blocked with 3% BSA in PBS and another uncoated well blocked as a control. Phages were added, incubated for 2 h at 37°C, unbound phages were washed away, and bound phages were detected with anti-M13 antibody conjugated to horseradish peroxidase (Pharmacia). The OD_{405} was measured after 1 h of 2,2-azino-di-3-ethylthiazoline sulfo-
rate reaction.

2.6. Mw finger print analysis of scFv-coding inserts

For each ELISA-positive clone, a 20-μl PCR mix was prepared adding 1 pmol LMB3 and TD-SEQ1 primers (Table 1E) [18]. Bacteria were transferred to the PCR mix and the following program was run: 10 min at 95°C followed by 30× (1 min at 94°C, 2 min at 55°C, and 2 min at 72°C). The reaction product was digested with MwI and analyzed by agarose gel electrophoresis.

2.7. Recombinant DNA techniques

Analysis of recombinant clones and DNA sequencing of the scFv-encoding sequences were carried out as described [19].

2.8. Expression of soluble scFv fragments in E. coli and Western blot analysis

Periplasmic extracts of recombinant E. coli HB2151 [17] containing a phENI-scFv phagemid were prepared according to the manufacturer’s instruction (Expression Module of the RPAS kit; Pharmacia). The total soluble protein concentration was determined with the Bio-Rad protein assay using BSA as a standard [14]. Periplasmic extracts were analyzed by Western blot using the anti-c-myc monoclonal antibody 9E10 [20] for scFv fragment detection (a kind gift from Dr. Arjen Schots, Landbouwuniversiteit Wageningen, The Netherlands) and anti-mouse antibodies, coupled to alkaline phosphatase (Sigma). The blots were developed using 5-bromo-4-chloro-3-indolyl phosphate (BCIP) as substrate and nitroblue tetrazolium chloride (NBT).

2.9. ELISA analysis using soluble scFv fragments

For each analyzed scFv fragment, a well was coated with 10 μg/ml DFRrec in PBS and blocked with 3% BSA in PBS whereas an uncoated well was blocked as a control. To both wells, 200 ng of each scFv fragment was added and incubated for 2 h at 37°C. The plates were washed and bound scFv fragments were detected using biotinylated anti-c-myc 9E10 monoclonal antibodies followed by streptavidin-conjugated alkaline phosphatase (Boehringer, Mannheim, Germany). The reaction, using 4-methylumbelliferyl phosphate as substrate, was followed fluorometrically and the F_{max} was calculated as ΔF/min reaction.

2.10. Western blot analysis of flower proteins using recombinant phages

Protein extracts were prepared from flower buds as described [13]. The protocol for Western blotting with recombinant phages was carried out as described [21]. Blots were incubated with 10 μg/ml recombinant phages and detected by a biotinylated anti-M13 antibody (5 PRIME⇒3 PRIME, Boulder, CO, USA) followed by streptavidin coupled to alkaline phosphatase. The blots were developed using BCIP as substrate and NBT.

3. Results

3.1. Overproduction of DFR in E. coli

To obtain a sufficient amount of protein for immunization, DFRrec-mediated recombinant phage selection, and subsequent scFv fragment characterization, DFR was overproduced in E. coli BL21(DE3) using the pET vector system [11]. DFRrec was expressed in high amounts (Fig. 1A), constituting at least 50% of total protein. However, more than 95% was obtained as inclusion bodies.

To purify the recombinant protein from E. coli lysates, the inclusion bodies were pelleted, solubilized in sample buffer, and separated by SDS-PAGE. Proteins were visualized by
protein recovered by gel elution. This approach resulted in

3.2. Construction of the phage display library

V^-back

Vx-front

V^-front

K

Vn-front

K-front and V^-front contain the coding sequence for the carboxyl-terminal end of the (Gly4Ser)3 linker (in italics) at their 5' end whereas V(Gly4Ser)3 linker (underlined) at their 5' end, respectively. Both the primer sets V^-back and V^-back contain the restriction sites

Spell Noti/SalI

K

The primer sets Vn-front and Vn-back contain the restriction sites (in italics) and the coding sequence for the amino-terminal end of the Sfil/Ncol.

fD-SEQ1 5'-GAATTTTCTGTATGAGG-3'

LMB3 5'-CAGGAAACACGTAGATGAC-3'

(E) Fingerprint analysis

5'-GCCCTCGAGACTAGTCGCGGCCGCGTCGAC-3'

MuPD18 5'-CTCGCGGCCCAGCCGGCAATGGCCGACGTCGAC-3'

(D) ScFv reamplification

MuPD15 5'-GCGGCCCAGCCGGCGCTAAGGTCGGGCGGCGGTCGACCAACGTC-3'

MuPD19 5'-GCGGCCCAGCCGGCGGTCGACCAACGTC-3'

(E) Fingerprint analysis

LMB3 5'-CAGGAAACACGTAGATGAC-3'

COH32 5'-GGCCAGTCGGTGCGTGGAC-3'

MuPD26 5'-AGCGGCGGTGGCGGTTCTGGAGGCGGCGGTTCTGGATCGAGTGTG-3'

MuPD21 5'-AGCGGCGGTGGCGGTTCTGGAGGCGGCGGTTCTGGATCGAGTGTG-3'

MuPD20 5'-AGCGGCGGTGGCGGTTCTGGAGGCGGCGGTTCTGGATCGAGTGTG-3'

MuPD37 5'-CTCGCGGCCCAGCCGGCGCTAAGGTCGGGCGGCGGTCGACCAACGTC-3'

MuPD34 5'-CTCGCGGCCCAGCCGGCGCTAAGGTCGGGCGGCGGTCGACCAACGTC-3'

MuPD28 5'-AGCGGCGGTGGCGGTTCTGGAGGCGGCGGTTCTGGATCGAGTGTG-3'

MuPD32 5'-ACTAGTCGGCGCGCGTCGACAGCTAGTCGGCGCGCGTCGACCTGACATG-3'

MuPD29 5'-AGCGGCGGTGGCGGTTCTGGAGGCGGCGGTTCTGGATCGAGTGTG-3'

MuPD27 5'-AGCGGCGGTGGCGGTTCTGGAGGCGGCGGTTCTGGATCGAGTGTG-3'

MuPD33 5'-GCCCTCGAGACTAGTCGCGGCCGCGTCGAC-3'

MuPD10 5'-AGCGGCGGTGGCGGTTCTGGAGGCGGCGGTTCTGGATCGAGTGTG-3'

MuPD11 5'-AGCGGCGGTGGCGGTTCTGGAGGCGGCGGTTCTGGATCGAGTGTG-3'

MuPD35 5'-AGCGGCGGTGGCGGTTCTGGAGGCGGCGGTTCTGGATCGAGTGTG-3'

MuPD22 5'-AGCGGCGGTGGCGGTTCTGGAGGCGGCGGTTCTGGATCGAGTGTG-3'

MuPD36 5'-AGCGGCGGTGGCGGTTCTGGAGGCGGCGGTTCTGGATCGAGTGTG-3'

MuPD31 5'-AGCGGCGGTGGCGGTTCTGGAGGCGGCGGTTCTGGATCGAGTGTG-3'

MuPD30 5'-AGCGGCGGTGGCGGTTCTGGAGGCGGCGGTTCTGGATCGAGTGTG-3'

MuPD12 5'-AGCGGCGGTGGCGGTTCTGGAGGCGGCGGTTCTGGATCGAGTGTG-3'

sodium acetate treatment, the DFR band was cut out and the protein recovered by gel elution. This approach resulted in purification of about 1 mg soluble DFR per gel. Theoretically, approximately 30 mg could be purified per liter induced culture. The purity of DFR was assessed by analytical SDS-PAGE (Fig. 1B). Some faint bands of contaminating protein were detectable and the purity was estimated to be at least 80%.

3.2. Construction of the phage display library

Seven female mice were hyper-immunized with DFR. Using ELISA with protein extracts from flower buds of *Petunia hybrida* RED27 (*An6*, wild type for DFR) and WHITE80 (*an6*, lacking DFR) as a control, it was shown that polyclonal sera from the DFR-immunized mice recognized native DFR from flower buds (data not shown). The spleens of two mice (one Balb/c and one C57BL/6) with high anti-DFR antibody titer (about 1:100000) were dissected and mRNA was purified from isolated cells. First-strand cDNAs were generated in four separate reverse transcriptase PCR reactions, using primers binding to constant Ig domains (Table 1B). Those amplified in four separate reverse transcriptase PCR reactions, using primers binding to constant Ig domains (Table 1B). Those amplified V regions were subsequently used for successful amplification of variable (V) regions of either IgG1 or IgG2a/b, heavy (VQ1 and VG2) or λ light chains (Vλ and VL) using extended sets of immunoglobulin-specific primers (Table 1C). Only VG1 and VG2 were amplified, because IgG1 and IgG2a/b constitute the major part (>90%) of IgGs in mouse serum. The amplified V regions were purified from the PCR reaction and analyzed by

### Table 1

<table>
<thead>
<tr>
<th>Primer sequences</th>
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<tbody>
<tr>
<td>(A) DFR cDNA amplification</td>
</tr>
<tr>
<td>5'-AAAGATCTGATCCTAGACGTTTTGACCTACACACGGTC-3'</td>
</tr>
<tr>
<td>5'-CCGGGATCCATGCATGGGCAAGTTCAT-3'</td>
</tr>
<tr>
<td>(B) First-strand cDNA synthesis</td>
</tr>
<tr>
<td>COH30 5'-GCGGACGCTGACCTAGACGTTTTGACCTACACACGGTC-3'</td>
</tr>
<tr>
<td>COH32 5'-AAAGATCTGATCCTAGACGTTTTGACCTACACACGGTC-3'</td>
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<tr>
<td>MuPD31 5'-AAAGATCTGATCCTAGACGTTTTGACCTACACACGGTC-3'</td>
</tr>
<tr>
<td>MuPD32 5'-AAAGATCTGATCCTAGACGTTTTGACCTACACACGGTC-3'</td>
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<tr>
<td>(C) Amplification of VH and VL domains</td>
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<tr>
<td>Vh-front</td>
</tr>
<tr>
<td>MuPD3 5'-GCGGACGCTGACCTAGACGTTTTGACCTACACACGGTC-3'</td>
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<tr>
<td>MuPD4 5'-GCGGACGCTGACCTAGACGTTTTGACCTACACACGGTC-3'</td>
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<td>MuPD5 5'-GCGGACGCTGACCTAGACGTTTTGACCTACACACGGTC-3'</td>
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<td>MuPD6 5'-GCGGACGCTGACCTAGACGTTTTGACCTACACACGGTC-3'</td>
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<td>MuPD7 5'-GCGGACGCTGACCTAGACGTTTTGACCTACACACGGTC-3'</td>
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<td>MuPD8 5'-GCGGACGCTGACCTAGACGTTTTGACCTACACACGGTC-3'</td>
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<td>MuPD9 5'-GCGGACGCTGACCTAGACGTTTTGACCTACACACGGTC-3'</td>
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<td>MuPD10 5'-GCGGACGCTGACCTAGACGTTTTGACCTACACACGGTC-3'</td>
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<td>MuPD11 5'-GCGGACGCTGACCTAGACGTTTTGACCTACACACGGTC-3'</td>
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<td>MuPD12 5'-GCGGACGCTGACCTAGACGTTTTGACCTACACACGGTC-3'</td>
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<tr>
<td>Vh-back</td>
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<td>MuPD13 5'-GCGGACGCTGACCTAGACGTTTTGACCTACACACGGTC-3'</td>
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<tr>
<td>MuPD14 5'-GCGGACGCTGACCTAGACGTTTTGACCTACACACGGTC-3'</td>
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<td>MuPD15 5'-GCGGACGCTGACCTAGACGTTTTGACCTACACACGGTC-3'</td>
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<td>MuPD16 5'-GCGGACGCTGACCTAGACGTTTTGACCTACACACGGTC-3'</td>
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<td>MuPD17 5'-GCGGACGCTGACCTAGACGTTTTGACCTACACACGGTC-3'</td>
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<td>MuPD18 5'-GCGGACGCTGACCTAGACGTTTTGACCTACACACGGTC-3'</td>
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<td>MuPD19 5'-GCGGACGCTGACCTAGACGTTTTGACCTACACACGGTC-3'</td>
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<td>MuPD20 5'-GCGGACGCTGACCTAGACGTTTTGACCTACACACGGTC-3'</td>
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<td>MuPD21 5'-GCGGACGCTGACCTAGACGTTTTGACCTACACACGGTC-3'</td>
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<td>MuPD22 5'-GCGGACGCTGACCTAGACGTTTTGACCTACACACGGTC-3'</td>
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<td>MuPD25 5'-GCGGACGCTGACCTAGACGTTTTGACCTACACACGGTC-3'</td>
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<tr>
<td>MuPD26 5'-GCGGACGCTGACCTAGACGTTTTGACCTACACACGGTC-3'</td>
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<tr>
<td>(D) ScFv reamplification</td>
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<tr>
<td>MuPD18 5'-CTCAGGCGAAGCGCAAAGCTACACACGGTC-3'</td>
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<tr>
<td>MuPD33 5'-GCCCTCGAGACTAGTCGCGGCCGCGTCGAC-3'</td>
</tr>
<tr>
<td>(E) Fingerprint analysis</td>
</tr>
<tr>
<td>LMB3 5'-CAGGAAACACGTAGATGAC-3'</td>
</tr>
<tr>
<td>IDSEQ1 5'-GAATTTTCTGTATGAGG-3'</td>
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</tbody>
</table>

The primer sets Vh-front and Vh-back contain the restriction sites SfiI/NcoI (in italics) and the sequencing code for the amino-terminal end of the (Gly4Ser)3 linker (in italics) whereas Vx-front and Vx-back contain the restriction sites Spel/Ncol (in italics) at their 5' end, respectively. Both the primer sets Vh-back and Vh-back contain the restriction sites Spel/Ncol and XhoI/Spel/Sall (in italics) at their 5' end.}

agarose gel electrophoresis (Fig. 2A). Yield and length of amplified fragments were similar for both mice. DNA fragments with an expected length of approximately 380 bp for V{sub}G1 and 360 bp for V{sub}k and V{sub}λ were obtained, although the main part of V{sub}k consisted of slightly longer fragments. For V{sub}G2, longer fragments of approximately 450 bp were obtained rather than fragments with the expected size of 380 bp. Most probably, these longer fragments resulted from the amplification by annealing of the front primers (Table 1C) and the remaining C{sub}H1- or C{sub}L-specific primer from the unpurified first-strand cDNA reaction, instead of the back primers (Table 1C). Between 100 and 400 ng of each fragment could be purified from a single PCR reaction. By this PCR reaction, parts of the (Gly{sub}4Ser){sub}3 linker sequence [16] were incorporated at the 5′ and 3′ site of variable light and heavy chain regions, respectively. The complementary (Gly{sub}4Ser){sub}3-coding sequence overlap of 21 bp enabled random assembly of scFv-encoding fragments by SOE-PCR avoiding three fragment PCR reactions [17]. ScFv-encoding fragments were assembled using equal amounts of V{sub}G1, V{sub}G2, V{sub}k and V{sub}λ. These assembled scFv-encoding fragments were reamplified to incorporate flanking S{sub}fi- and NotI-cloning sites at the 5′ and 3′ ends, respectively. The reamplification reactions were analyzed by agarose gel electrophoresis (Fig. 2B). Again yield and length of the reaction product were similar for both mice. DNA fragments with expected length of approximately 750 bp were obtained. An additional, slightly shorter faint band was also detected. About 200 ng amplified fragment was obtained per reaction. This means that with the purified reaction products of one set of amplified V regions, at least 5 μg of scFv-encoding fragments were obtained to establish a phage display library. The amplified PCR products were purified, digested with S{sub}fi and NotI, and ligated in the phage display vector pHENI [17], resulting in a library of approximately 1.2×10{sup}6 independent recombinant clones, containing inserts of approximately 750 bp.

Fig. 2. A: Agarose gel analysis of PCR-amplified variable (V) regions from two DFR{sup}rec-immunized mice (C57BL/6 and Balb/c). In four separate PCR reactions, the V regions of IgG{sub}1 (G1) and IgG{sub}2a,b (G2) heavy and κ and λ light chains were amplified and purified by agarose gel electrophoresis. One tenth of each purified PCR product was analyzed on a 1.5% gel. B: Agarose gel analysis of the assembly and reamplification of scFv-encoding fragments from two DFR{sup}rec-immunized mice (C57BL/6 and Balb/c). For each mouse, one tenth of five SOE amplification reactions was analyzed on a 0.8% agarose gel. M, molecular weight marker.

3.3. Isolation of DFR{sup}rec-binding scFv fragments

The phage display library was enriched for DFR{sup}rec-binding clones by several rounds of panning against DFR{sup}rec. As a control, panning was carried out against BSA. The number of eluted phages after each panning was determined (Fig. 3A). In the second panning against DFR{sup}rec, the number of eluted phages was not significantly higher than that in the control panning. However, the third panning round resulted in an almost 2000-fold enrichment of DFR-eluted phages compared to that of the BSA control. This indicated a specific enrichment for DFR-binding phages. Forty monoclonal phage stocks were prepared both from the original library and the enriched libraries after each panning step and screened for DFR-binding in phage ELISA. After the second panning, al-
3. Characterization of isolated scFv fragments

Sequencing of the scFv-encoding regions confirmed the five different fingerprints and resulted in the amino acid sequences A1, A3, A4, G4, and H3 shown in Fig. 4B. Amino acid sequence alignment showed that three of the scFv fragments contained the same H3 loop. This suggests that these three different scFv proteins recognize a similar epitope of DFR<sub>rec</sub>. Two scFv fragments (A1 and A4) lack seven amino acids at the carboxyl-terminal end of the light chain, amino-terminally of the c-myc tag, presumably due to artifacts in the PCR reactions, but without impairing the functionality of these scFv fragments nor their expression efficiency in E. coli. Alignment of the scFv fragments with the Kabat database [22] revealed that the VH domains of all five scFv fragments belong to murine V<sub>H</sub> family II, whereas the VL domains of scFv fragments A<sub>1</sub>, A<sub>3</sub>, and H<sub>3</sub> and of scFv fragments G<sub>4</sub> and H<sub>4</sub> belong to family I and family III of murine K light chains, respectively.

For expression analysis of soluble scFv fragments, the five identified phagemids were transformed in E. coli strain HB2151 [17]. Periplasmic extracts were isolated 3 h after induction and analyzed on Western blot using the anti-c-myc antibody 9E10 [20] for detection (Fig. 5). A single band of approximately 32 kDa for A1, 34 kDa for A3 and A4, and 35 kDa for G4 and H3 was detectable. As observed by others [16,23], the molecular weights calculated for the processed scFv fragments (28.11 for A1; 28.62 for A3; 28.55 for A4; 28.96 for G4; and 28.84 for H3) are less than indicated by gel migration, probably due to aberrant migration behavior during SDS-PAGE [24]. All five soluble scFv fragments were able to specifically bind to DFR<sub>rec</sub> in ELISA (Table 2). ScFv frag-
ments A1, A3, A4 and H3 gave similar ELISA signals, whereas the scFv fragment G4 gave a lower signal (Table 2). It was found by dot blot that the low signal was not due to a lower detection efficiency of G4 by anti-c-myc antibody but most probably to a lower binding affinity for DFR in this ELISA. The complementarity-determining regions (CDRs) H1, H2, H3, L1, L2, and L3 in VL are indicated as are the (Gly, Ser) linker and the carboxyl-terminal c-myc tag. Classification of CDRs was carried out as described by Kabat [22].

![Fig. 4. A: Fingerprint analysis of five clones (A1, A3, A4, G4, and H3) binding to DFR. SCFv inserts were amplified by PCR, digested with MvaI and separated on a 2% agarose gel. B: Amino acid sequence alignment of the five different isolated SCFv fragments. The complementarity-determining regions (CDRs) H1, H2, and H3 in VH and L1, L2, and L3 in VL are indicated as are the (Gly, Ser) linker and the carboxyl-terminal c-myc tag. Classification of CDRs was carried out as described by Kabat [22].](image)

SA. The same result was obtained in phage ELISA for detection of DFR (results not shown).

Finally, the binding of all five scFv fragments to DFR extracted from flowers and the cross-reactivity with other plant proteins was investigated by Western blot and ELISA. Protein extracts from flower buds of *Petunia hybrida* varieties RED27 and WHITE80 were prepared. For each scFv, a Western blot was performed using scFv monochonal phages as detecting agents (Fig. 6). All five scFv phages revealed a single protein band at the expected size of 42.5 kDa in extracts from RED27, and none with the WHITE80 extract. This result shows that the five scFv fragments specifically recognize DFR in RED27 flower extracts and show no cross-reactivity to other extracted plant proteins. By ELISA using recombinant phages at least three out of five isolated scFv fragments (A1, A3, and G4) were found to bind native DFR protein from flower buds (data not shown).

### 4. Discussion

We successfully generated a set of scFv fragments that bind to DFR from *Petunia hybrida* by using phage display technology. We were able to isolate five different DFR-binding scFv fragments from a phage display library consisting of 1.2×10^6 recombinant clones. This library was constructed from spleen mRNA of mice, immunized with recombinant DFR. As compared to earlier reports [7,10], an optimized and extended set of primers for murine scFv phage display library construction (Table 1B) was used, which contain parts of the (Gly, Ser) linker sequence, allowing SOE-PCR with two instead of three DNA fragments. We demonstrated that all five scFv fragments, either displayed on phages or as soluble protein, specifically recognize DFR in ELISA. Moreover, all

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Reactivity of soluble expressed scFv fragments in ELISA</th>
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<tr>
<td></td>
<td>A1</td>
</tr>
<tr>
<td>DFR</td>
<td>0.957</td>
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<tr>
<td>BSA</td>
<td>0.002</td>
</tr>
</tbody>
</table>

For each scFv (A1, A3, A4, G4 and H3) a well was coated with DFR or BSA as a control. Equal amounts of the scFv fragments were added to each well and detected by the c-myc tag-specific 9E10 monoclonal antibody. For each well the V_max is given as AU/min substrate reaction.
five recombinant phage clones bind to DFR, extracted from flower buds from the Petunia variety RED27 on Western blot, and no cross-reactivity with other proteins was observed. At least three of the five scFv fragments also recognize native DFR, extracted from flower buds, as determined by ELISA. So far, only the strongest DFR-binding phage clones were further characterized. However, by analyzing more clones from the library after the second panning, additional but weaker DFR-binding phage clones could be found.

The generated recombinant scFv antibodies were used successfully as detecting agent in ELISA and Western blot and, given their specificity in both assays, they may also be suitable for cytological immunodetection and immunoaffinity chromatography. Until now, most researchers in plant molecular biology have used polyclonal antibodies, mainly because they are easy to develop. However, in many cases, recombinant antibodies from phage display libraries may be a better choice because they are derived from a single E. coli clone, increasing the chances for the antibody to be specific and, consequently, decreasing problems associated with cross-reactivity. Moreover, they can be produced in almost unlimited quantities [25].

We intend to use the five DFR-binding scFv fragments to study the intracellular immunomodulation of DFR in plants by interfering in the metabolic flavonoid pathway. Because of the coselection of scFv fragments with their encoding sequences, the generation of chimeric gene constructs can immediately be started for stable expression in the appropriate cell compartment of Petunia hybrida. ScFv-mediated immunomodulation in purple wild-type varieties should be detectable at the phenotypical level due to the white flower color caused by the inhibition of DFR activity. Moreover, this should allow the analysis of scFv functionality and stability in transgenic Petunia hybrida plants.

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