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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 V_H and V_L 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.

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 knock-out 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. Overproduction 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 *Bam*HI and a *Bgl*II restriction site at the 5′ and 3′ end, respectively. The PCR fragment was cloned in a *Sma*I-linearized pGEM2 cloning vector and confirmed by sequencing. The *Bam*HI/*Bgl*II cDNA fragment was cloned in-frame into the *Bam*HI-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(DE3) 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 OD_{600} of 0.4. Recombinant DFR (DFR^{rec}) expression was induced by adding 1 mM isopropyl β-D-thiogalactopyranoside and analyzed by SDS-PAGE.

2.2. Purification of DFR^{rec} from E. coli extracts

A DFR^{rec}-producing BL21(DE3) 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 McIlvain C buffer [13], containing 1 mM phenylmethylsulfonyl fluoride, and so-

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Abbreviations: BSA, bovine serum albumin; cfu, colony-forming units; C_H1, constant region 1 of heavy chain; C_L, constant region of light chain; DFR, dihydroflavonol 4-reductase; DFR^{rec}, recombinant DFR; ELISA, enzyme-linked immunosorbent assay; FU, 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; V, variable domain; V_H, variable region of heavy chain; V_L, variable region of light chain; $V_κ$, variable region of κ light chain; $V_λ$, variable region of λ light chain; V_{G1}, variable region of IgG_1 heavy chain; V_{G2}, variable region of $IgG_{2a/b}$ heavy chain

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nicated. Inclusion bodies were pelleted (13 000×*g*/30 min/4°C), dissolved in SDS-PAGE loading buffer, boiled for 10 min, put on ice, centrifuged for 10 min at 10 000×*g* and separated on a preparative 10% polyacrylamide gel. After staining of the gel in 4 M sodium acetate for 30 min, the DFR band was cut out and incubated for 3×10 min in SDS-PAGE running buffer. Proteins were eluted from the gel using a Model 422 Electro-Eluter (Bio-Rad, Hercules, CA, USA), extensively dialyzed against PBS, and their concentration was determined by UV spectrophotometry. The purity of DFR^{rec} was analyzed by analytical SDS-PAGE.

2.3. Immunization of mice, spleen dissection and mRNA purification

Primary injections were done intramuscularly using 32 μg DFR^{rec} and Hunter's Titer Max adjuvant (Sigma, St. Louis, MO, USA) [15]. Two months later, mice were boosted with 18 μg DFR^{rec} in PBS, without adjuvant. The anti-DFR^{rec} antibody titer was determined by indirect ELISA [15]. Mice showing a high immune response were boosted intravenously with 32 μg DFR^{rec} in PBS 2 months later. Five days after this boost, spleen cells were prepared [15] for mRNA purification using the Fasttrack mRNA Kit (Invitrogen, San Diego, CA, USA).

2.4. Construction of the phage display library

First-strand cDNA was synthesized in four separate reactions using the first-strand cDNA synthesis kit from Pharmacia (Uppsala, Sweden) and the primers COH30, COH32, MuPD31, and MuPD32 (Table 1B), binding to the C_H1 region of IgG_1 and $IgG_{2a/b}$ heavy chains and C_L region of κ and λ light chains, respectively. Each reaction contained 100 ng spleen mRNA and one of these primers at a final concentration of 2 pmol/μl.

PCR amplification of the variable regions was done using the following specific sets of primers binding to murine immunoglobulin variable regions of heavy (V_H) and light chains ($V_κ$ and $V_λ$) (Table 1C): V_H-front (MuPD3-12 mix) and V_H-back (MuPD34-37 mix), $V_κ$-front (MuPD19-23 mix) and $V_κ$-back (MuPD27-29 mix), and $V_λ$-front (MuPD26) and $V_λ$-back (MuPD30). PCR reactions were done in a total volume of 50 μl by adding 20 pmol of each primer set, a complete first-strand cDNA reaction and using the following program: 30× (1 min at 94°C, 2 min at 60°C, and 2 min at 72°C). PCR products were separated by agarose gel electrophoresis, purified from the gel and used for scFv assembly by splice-overlap extension PCR (SOE-PCR). Equal amounts of IgG_1 and $IgG_{2a/b}$ heavy chain and κ and λ light chain variable regions were mixed (12.5 ng of each) and the following program was run: 7× (5 min at 94°C, 2 min at 55°C, and 10 min at 72°C). One fifth of the SOE-PCR was used for scFv fragment reamplification with primers MuPD18 and MuPD33 (Table 1D). Reamplifications were done in a total volume of 50 μl by adding 2.5 pmole of each primer and using the following program: cycle 1, 1× (15 min at 95°C); cycle 2, 30× (2 min at 60°C, 3 min at 72°C, and 1 min at 94°C); and cycle 3, 1× (2 min at 60°C and 10 min at 72°C). Amplified fragments were purified using S-400 sephacryl microspin columns (Pharmacia), concentrated by precipitation, digested using *Sfi*I and *Not*I and ligated into the phage display vector pHENI [17]. Transformation by electroporation was done using the highly competent *E. coli* strain MC1061F′ (a gift from Prof. Dr. E. Remaut, Laboratory of Molecular Biology, Universiteit Gent). Recombinant clones were selected on SOBAG medium, containing 2% glucose and 100 μg/ml carbenicillin. Plasmid DNA was prepared from the complete library and transformed by electroporation in the SupE *E. coli* strain TG1 for phage display [17].

2.5. Enrichment and screening of the phage display library

A phage library was prepared from a TG1 library culture upon infection with M13KO7 helper phage (Pharmacia) and the titer was determined. An immunotube (Nunc, Gibco BRL, Gaithersburg, MD, USA) was coated overnight at 4°C with 2.5 ml of a 0.5 μg/ml DFR^{rec} solution in PBS and blocked with 4 ml of 3% BSA in PBS. A control panning was done in parallel in an uncoated, blocked immunotube. Phages (2.5 ml containing $1–5×10^{12}$ cfu) were added and incubated for 2 h at 37°C. Unbound phages were removed by washing 20 times with PBS and 20 times with PBS+0.1% Tween. Antigen-bound phages were eluted by adding 1 ml of 1 M triethylamine for 10 min and neutralized with 1 ml of 1 M Tris-HCl (pH 7.4). Eluted phages were reinfected in TG1, plated on SOBAG medium containing carbenicillin and the titer was determined.

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 DFR^{rec} 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 antibodies conjugated to horseradish peroxidase (Pharmacia). The OD_{405} was measured after 1 h of 2,2-azino-di-3-ethylbenz-thiazoline sulfonate reaction.

2.6. MvaI fingerprint analysis of scFv-coding inserts

For each ELISA-positive clone, a 20-μl PCR mix was prepared adding 50 pmol LMB3 and fD-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 *Mva*I 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 *p*-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 DFR^{rec} 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 V_{max} was calculated as ΔFU/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^{11} cfu/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, DFR^{rec}-mediated recombinant phage selection, and subsequent scFv fragment characterization, DFR was overproduced in *E. coli* BL21(DE3) using the pET vector system [11]. DFR^{rec} 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

Table 1
Primer sequences

(A) DFR cDNA amplification	
	5′-AAAGATCTGACTTCAACATTGCTTAACATTTCTG-3′
	5′-CGCGGATCCATGGCAAGTGAAGCAGTTCAT-3′
(B) First-strand cDNA synthesis	
COH30	5′-GGCCAGTGGATAGACAGA-3′
COH32	5′-TAACCCTTRACCAGGCATCC-3′
MuPD31	5′-ACTAGTCGCGGCCGCGTCGACGGATACCGTTGGTGCAGCATCAGC-3′
MuPD32	5′-ACTAGTCGCGGCCGCGTCGACAAGCTCYTCAGAGGAAGGTGGAAA-3′
(C) Amplification of V_H and V_L domains	
V_H-front	
MuPD3	5′-*GCGGCCCAGCCGGCCATGG*CCGAKGTRCAGCTTCAGGAGTCRGGA-3′
MuPD4	5′-*GCGGCCCAGCCGGCCATGG*CCCAGGTGCAGCTGAAGSAGTCWGGM-3′
MuPD5	5′-*GCGGCCCAGCCGGCCATGG*CCSAGGTYCAGCTGCARCAGTCWGGD-3′
MuPD6	5′-*GCGGCCCAGCCGGCCATGG*CCSAGGTCCARCTGCAGSARYCTGGR-3′
MuPD7	5′-*GCGGCCCAGCCGGCCATGG*CCGAGGTTCAGCTGCAGCAGTCTGGG-3′
MuPD8	5′-*GCGGCCCAGCCGGCCATGG*CCGARGTGAAGCTGGTGGARTCTGGR-3′
MuPD9	5′-*GCGGCCCAGCCGGCCATGG*CCGAGGTGAAGCTTCTCGAGTCTGGA-3′
MuPD10	5′-*GCGGCCCAGCCGGCCATGG*CCGARGTGAAGCTKGAKGAGWCTGR-3′
MuPD11	5′-*GCGGCCCAGCCGGCCATGG*CCGAVGTGMWGCTKGTGGAGTCTGGK-3′
MuPD12	5′-*GCGGCCCAGCCGGCCATGG*CCSAGGTYCAGCTKCAGCAGTCTGGA-3′
V_H-back	
MuPD34	5′-TCCAGAACCGCCACCGCCGCTACCGCCGCCACCTGMRGAGACDGTGASMGTRGTC-3′
MuPD35	5′-TCCAGAACCGCCACCGCCGCTACCGCCGCCACCTGMRGAGACDGTGASMGTRGTG-3′
MuPD36	5′-TCCAGAACCGCCACCGCCGCTACCGCCGCCACCTGMRGAGACDGTGASCAGRGTC-3′
MuPD37	5′-TCCAGAACCGCCACCGCCGCTACCGCCGCCACCTGMRGAGACDGTGASTGARGTT-3′
V_κ-front	
MuPD19	5′-AGCGGCGGTGGCGGTTCTGGAGGCGGCGGTTCTGACATTGTGMTGWCACAGTC-3′
MuPD20	5′-AGCGGCGGTGGCGGTTCTGGAGGCGGCGGTTCTGATRTTKTGATGACCCARAC-3′
MuPD21	5′-AGCGGCGGTGGCGGTTCTGGAGGCGGCGGTTCTRAMATTGTGMTGACCCAATC-3′
MuPD22	5′-AGCGGCGGTGGCGGTTCTGGAGGCGGCGGTTCTSAAAWTGTKCTSACCCAGTC-3′
MuPD23	5′-AGCGGCGGTGGCGGTTCTGGAGGCGGCGGTTCTGAYATYCAGATGACMCAGWC-3′
V_κ-back	
MuPD27	5′-*ACTAGTCGCGGCCGCGTCGAC*AGCMCGTTTCAGYTCCARYTT-3′
MuPD28	5′-*ACTAGTCGCGGCCGCGTCGAC*AGCMCGTTTKATYTCCARYTT-3′
MuPD29	5′-*ACTAGTCGCGGCCGCGTCGAC*AGCMCGTTTBAKYTCTATCTTTGT-3′
V_λ-front	
MuPD26	5′-AGCGGCGGTGGCGGTTCTGGAGGCGGCGGTTCTCARSYTGTKSTSACTCAGKMATCT-3′
V_λ-back	
MuPD30	5′-*ACTAGTCGCGGCCGCGTCGAC*CTGRCCTAGAGCAGTSASYTTGGT-3′
(D) ScFv reamplification	
MuPD18	5′-CTCGC*GGCCCAGCCGGCCATGG*CC-3′
MuPD33	5′-GCC*CTCGAGACTAGTCGCGGCCGCGTCGAC*-3′
(E) Fingerprint analysis	
LMB3	5′-CAGGAAACAGCTATGAC-3′
fD-SEQ1	5′-GAATTTTCTGTATGAGG-3′

The primer sets V_H-front and V_H-back contain the restriction sites *SfiI*/*NcoI* (in italics) and the coding sequence for the amino-terminal end of the $(Gly_4Ser)_3$ linker (underlined) at their 5′ end, respectively. Both the primer sets V_κ-back and V_λ-back contain the restriction sites *SpeI*/*NotI*/*SalI* (in italics) whereas V_κ-front and V_λ-front contain the coding sequence for the carboxyl-terminal end of the $(Gly_4Ser)_3$ linker (in italics) at their 5′ end [16]. Primers MuPD18 and MuPD33 contain the restriction sites *SfiI*/*NcoI* and *XhoI*/*SpeI*/*NotI*/*SalI* (in italics), respectively.
B = C/G/T, D = A/G/T, K = G/T, M = A/C, R = A/G, S = C/G, W = A/T, Y = C/T.

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^{rec} per gel. Theoretically, approximately 30 mg could be purified per liter induced culture. The purity of DFR^{rec} 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^{rec}. 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^{rec}-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^{rec} antibody titer (about 1:100 000) 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 were subsequently used for successful amplification of variable (V) regions of either IgG_1 or $IgG_{2a/b}$ heavy (V_{G1} and V_{G2}) or κ or λ light chains (V_κ and V_λ) using extended sets of immunoglobulin-specific primers (Table 1C). Only V_{G1} and V_{G2} were amplified, because IgG_1 and $IgG_{2a/b}$ constitute the major part (>90%) of IgGs in mouse serum. The amplified V regions were purified from the PCR reaction and analyzed by

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_{G1} and 360 bp for V_κ and V_λ were obtained, although the main part of V_λ consisted of slightly longer fragments. For V_{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_H1- or C_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 $(Gly_4Ser)_3$ linker sequence [16] were incorporated at the 5′ and 3′ site of variable light and heavy chain regions, respectively. The complementary $(Gly_4Ser)_3$-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_{G1}, V_{G2}, V_κ and V_λ. These assembled scFv-encoding fragments were reamplified to incorporate flanking *Sfi*I- and *Not*I-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 *Sfi*I and *Not*I, and ligated in the phage display vector pHENI [17], resulting in a library of approximately 1.2×10^6 independent recombinant clones, containing inserts of approximately 750 bp.

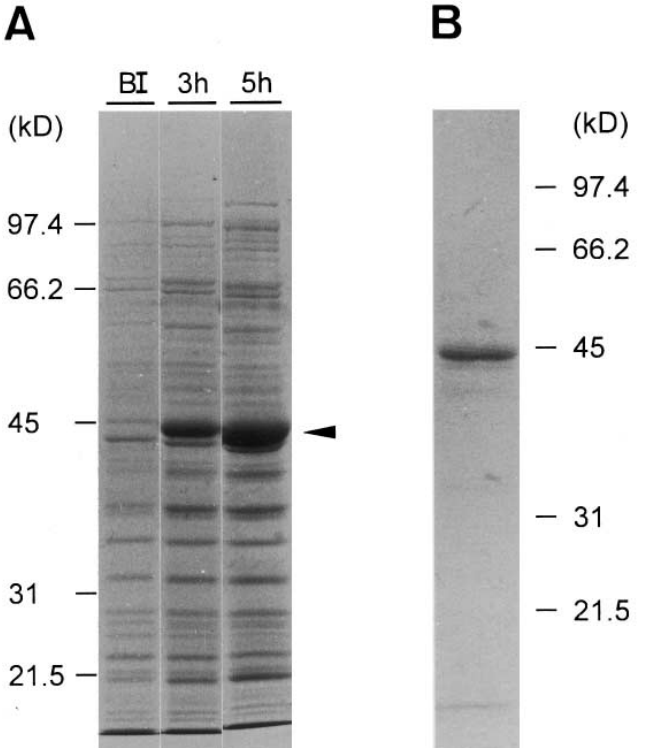


Fig. 1. A: SDS-PAGE analysis of DFR overproduction in *E. coli*. 1-ml culture samples were taken before induction at time zero (BI) and 3 and 5 h after induction. Bacteria were pelleted and boiled in gel loading buffer. One fourth of each protein extract was separated on a 10% polyacrylamide gel and proteins visualized by Coomassie blue staining. The arrow indicates the 44.5-kDa DFR^{rec} band. B: SDS-PAGE analysis of DFR^{rec}, purified from *E. coli* inclusion bodies by gel elution. 1 μg of purified protein was separated on a 10% polyacrylamide gel and visualized by Coomassie blue staining.

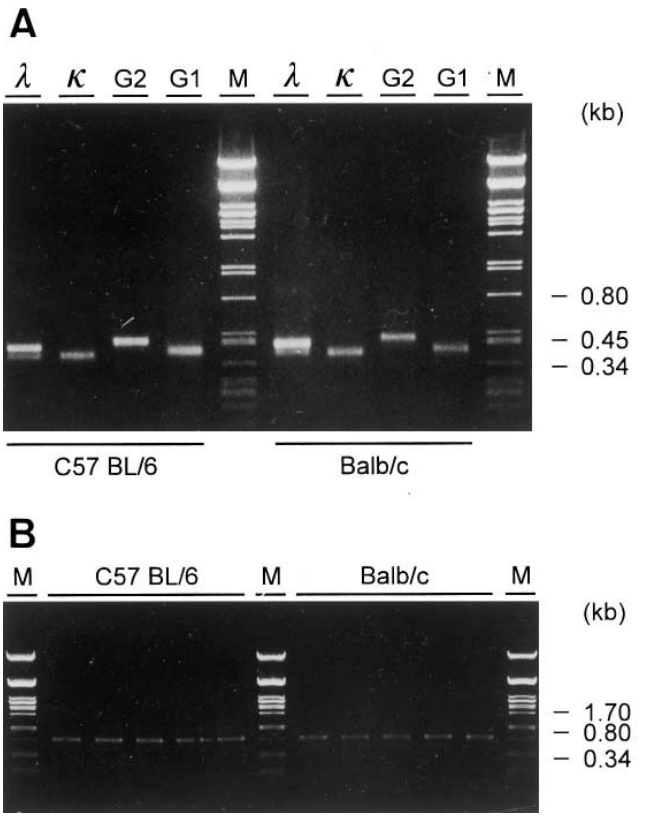


Fig. 2. A: Agarose gel analysis of PCR-amplified variable (V) regions from two DFR^{rec}-immunized mice (C57BL/6 and Balb/c). In four separate PCR reactions, the V regions of IgG_1 (G1) and $IgG_{2a/b}$ (G2) heavy and κ and λ light chains were amplified and purified by agarose gel electrophoresis. One 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^{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^{rec}-binding scFv fragments*

The phage display library was enriched for DFR^{rec}-binding clones by several rounds of panning against DFR^{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^{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-

A

PANNING (INPUT)	DFR^{rec}	BSA (CONTROL)
1 (5 x 10^{12} cfu)	2.6 x 10^5 cfu	1.3 x 10^5 cfu
2 (5 x 10^{12} cfu)	5.1 x 10^4 cfu	2.1 x 10^4 cfu
3 (1 x 10^{12} cfu)	1.9 x 10^9 cfu	9.9 x 10^5 cfu

B

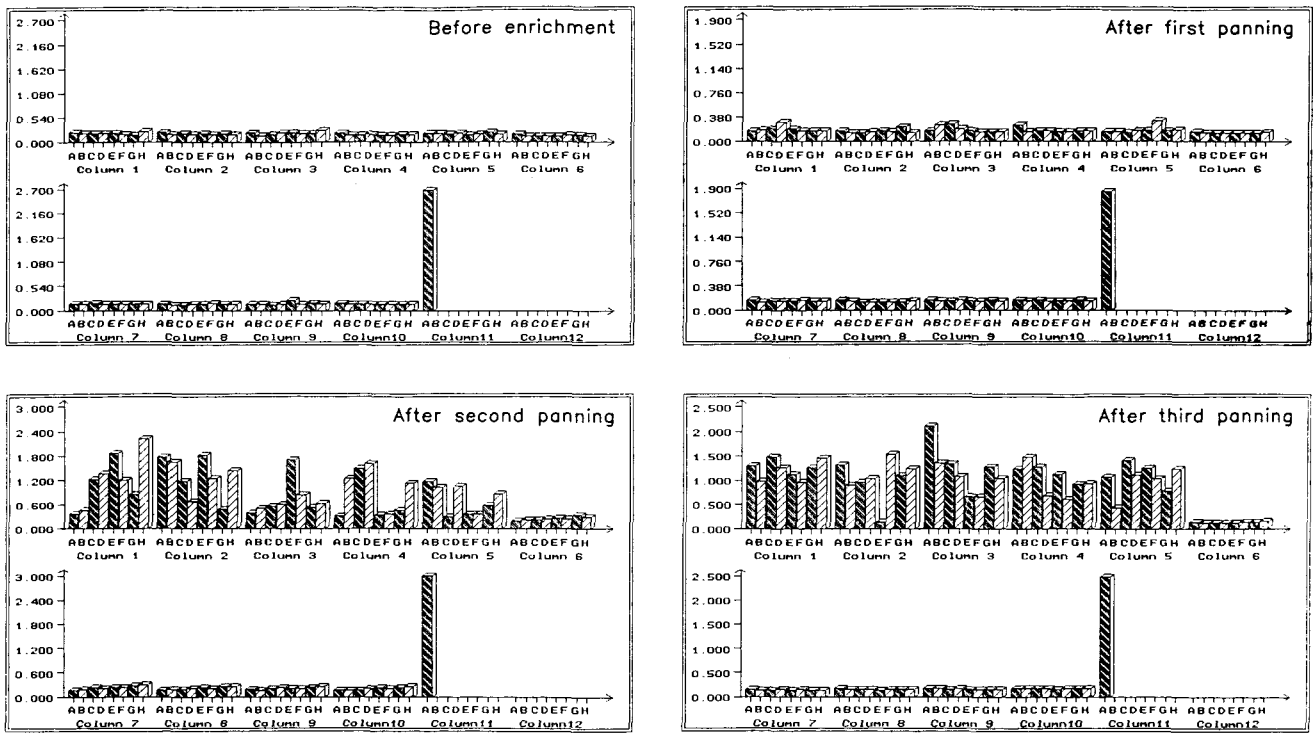


Fig. 3. A: Enrichment of the phage display library after three rounds of panning against DFR^{rec} (middle column). As a control, panning was done against BSA (right column). The left column shows the phage input in each panning step. Values in the middle and right columns represent the titer of eluted phages after each panning round. B: Colony screening by phage ELISA against DFR^{rec} from the library before enrichment and the enriched libraries after first, second, and third panning. Forty clones from each library were analyzed for DFR^{rec} binding. Eight wells (A–H) from columns 1, 2, 3, 4, and 5 were coated with DFR^{rec} and eight wells (A–H) from columns 6, 7, 8, 9, and 10 with BSA as negative control prior to addition of monoclonal phage clones. Well A from column 11 is a positive control. Wells B–H from column 11 and A–H from column 12 were not used. For each well, the OD_{405} is given after 1 h of substrate reaction.

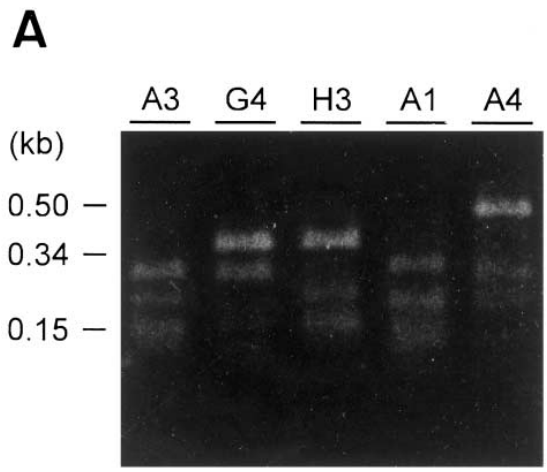
ready more than half of the tested clones scored positive (Fig. 3B), although the amount of eluted phages was only slightly higher than for the control (Fig. 3A). After the third panning, 39 clones scored positive for DFR^{rec} (Fig. 3B). *Mva*I fingerprint analysis of 40 random DFR^{rec}-binding clones, taken from the library after the third panning, revealed that based on the different fingerprints at least five different scFv fragments binding to DFR^{rec} were selected after three panning rounds from the established phage library (Fig. 4A). Four additional DFR^{rec}-binding clones from the library after the second panning, which gave high signals in phage ELISA, were analyzed, but no new clones were found.

3.4. 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^{rec}. 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 V_H domains of all five scFv fragments belong to murine V_H family II, whereas the V_L domains of scFv fragments A_1, A_3, and H_3 and of scFv fragments G_4 and A_4 belong to family I and family III of murine κ 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^{rec} 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^{rec} in this ELISA. The same result was obtained in phage ELISA for detection of DFR^{rec} (results not shown).



B

```
    1                                                   50
A1  MVEVQLQQSG AELVRPGASV TLSCKASGYT FTDYEMHWVK QTPVHGLEWI
A3  MVQVQLKQSG PELVRPGASV TLSCKASGYA FTAYNMHWVK QTPVHGLEWI
A4  MAEVQLQQSG PELVKPGASV KMSCKASGYS FGGYFMNWVK QSHGKSLEWI
G4  MVQVQLQQSG DDLVKPGASV KLSCKASGYT FTSYWINWIK QRPGQGLEWI
H3  MVQVQLKQSG AEQVRPGASV TLSCKASGYT FTGYEMHWVK QTPVHGLEWL
                                        H1
    51                                                  100
A1  GAVDPKSGVT AYNQKFKGKV RLTADKSSNT AYMELRSLTS EDSAVYYCTS
A3  GAIDPETGGI AYNQKFKGKA KLTADKSSST AYMELRSLTS EDSAIYYCTS
A4  GRINPYSGDT FYNQKFKDKA ILTVDKSSST AHMELLSLTS EDSAVYYCGR
G4  GRIAPGSGST YYNEMFKGKA TLTVDHSSST AHMELLSLTS DDSAVYYCVR
H3  GAIDPKSGVT AYNQKFKGKA RLTADKSSST AYMELRSLTS EDSAVYYCTS
        H2
    101                                                 150
A1  ......QIDY WGQGTTLTVS SGGGGSGGGG SGGGGSEIVL TQSPSSLATS
A3  ......QIDY WGQGTTLTVS AGGGGSGGGG SGSGGSDIVM TQSPSSLAVS
A4  RDYATGGINY WGQGTTLTVS SGGGGSGGGG SGGGGSDIQM TQSPASLTVS
G4  GDYGYGGINY WGQGTTLTVS SGGGGSGGGG SGGGGSDIVL SQSPASLAVT
H3  ......QIDY WGQGTTLTVS SGGGGSGGGG TGGGGSDIQM TQSPSSLLLS
      H3                      LINKER
    151                                                 200
A1  VGQKVTMNCK SSQSLLNISN QKNYLAWYQL KPGQSPKLLV YFASTRESGV
A3  VGQKVTMSCK SSQSLLNISN QKNYLAWYQK KPGQSPRLLV YFASTRESGV
A4  LGQKATISCR ASKSVFTSGN ..NYMHWYQQ KPGQPPKLLI SLASNLESGV
G4  QGQRATISCR ASKSVSTSGY ..SYLHWYQQ KPGQPPKLLI SLASNLESGV
H3  VGQKVTMSCK SSQSLLNSPK QKNYLAWYQL KPGQSPKLLV YFASTRESGV
                L1                                  L2
    201                                                 250
A1  PDRFIGSGSG TDFTLTISSV QSEDLADYFC QQHYNTPLTF GAGTKLELKR
A3  PNRFIGSGSG TDFTLTISSV QAEDLADYFC QQHYSIPLTF GAGTKLELKR
A4  PARFSGSGSE TDFTLNIHPV EEEDAATYYC QHNRKLPLTF GSGTKLELKR
G4  PTRFSGSGSG TDFTLNIHPV EEEDAAIYYC QHNRELPLTF GSGTKLEIKR
H3  PDRFIDSGSG TDFTLTITSV QPEDLADYFC QQHYSIPLTF GAGTKLELKR
                                        L3
    251                 277
A1  A.......VD AAAEQKLISE EDLNGAA
A3  ADAAPTVSVD AAAEQKLISE EDLNGAA
A4  A.......VD AAAEQKLISE EDLNGAA
G4  ADAAPTVSVD AAAEQKLISE EDLNGAA
H3  ADATPTVSVD AAAEQKLISE EDLNGAA
                c-myc TAG
```

Fig. 4. A: Fingerprint analysis of five clones (A1, A3, A4, G4, and H3) binding to DFR^{rec}. ScFv inserts were amplified by PCR, digested with *Mva*I and separated on a 2% agarose gel. B: Amino acid sequence alignment of the five different isolated scFv fragments. The complementarity-determining regions (CDRs) H_1, H_2 and H_3 in V_H and L_1, L_2 and L_3 in V_L are indicated as are the $(Gly_4Ser)_3$ linker and the carboxyl-terminal c-*myc* tag. Classification of CDRs was carried out as described by Kabat [22].

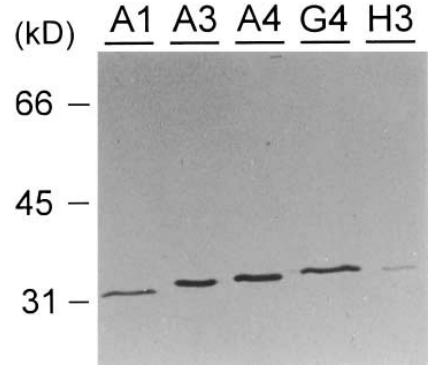


Fig. 5. Immunoblot of periplasmic proteins (1 μg) from HB2151 *E. coli* cells transformed with pHENI containing A1, A3, A4, G4 and H3 scFv-encoding sequences. The scFv fragments were detected with the c-*myc* tag-specific 9E10 monoclonal antibody.

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 monoclonal 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 (A_1, A_3, and G_4) 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^{rec}. 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 $(Gly_4Ser)_3$ 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^{rec} in ELISA. Moreover, all

Table 2
Reactivity of soluble expressed scFv fragments in ELISA

	A1	A3	A4	G4	H3
DFR^{rec}	0.957	0.991	1.187	0.203	1.201
BSA	0.002	0.002	0.002	0.003	0.003

For each scFv (A1, A3, A4, G4 and H3) a well was coated with DFR^{rec} 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 monoconal antibody. For each well the V_{max} is given as ΔFU/min substrate reaction.

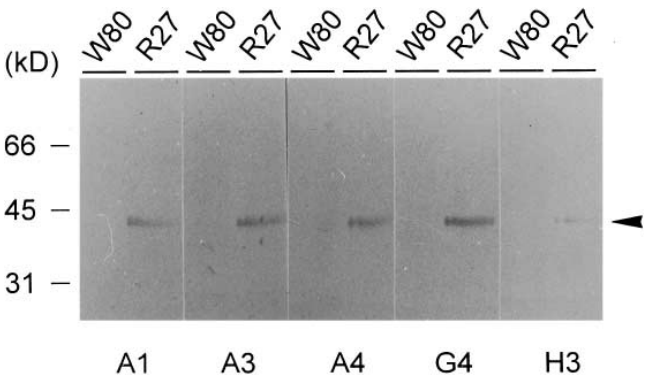


Fig. 6. Immunoblots of flower bud protein extracts (25 μg) from *Petunia hybrida* wild type RED27 and mutant WHITE80 varieties. Each of the five blots was developed with one of the five isolated scFv fragment phage clones. The arrow indicates the 42.5-kDa DFR band.

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^{rec}-binding phage clones were further characterized. However, by analyzing more clones from the library after the second panning, additional but weaker DFR^{rec}-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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