

**A NOVEL APPLICATION OF PHAGE DISPLAY
TECHNOLOGY FOR ANTIBODY SELECTION,
USING ALTERNATING ROUNDS OF POSITIVE
AND NEGATIVE SELECTION**

**Ivanaela Albanova, Lyuben Zagorchev[✉], Mariela Odjakova,
Miroslav Marinov, Denitsa Teofanova**

Received on March 2, 2025

Presented by N. Atanassova, Corresponding Member of BAS, on April 29, 2025

Abstract

Antibodies are a powerful tool in various studies concerning the expression and localization of proteins in response to various factors of the environment. Phage display technology offers a useful alternative to classical immunization approaches, as it does not require immunogenicity of the antigen, as well as the handling of live animals. However, the requirement for the antigen to be presented and isolated in a relatively pure form, and large quantity remains a major obstacle. In the present study, we offered a novel approach toward exploiting phage display antibody libraries for the fast selection of specific antibodies by alternating rounds of positive and negative selection. The overall aim was to select antibodies against antigens, presented in the treatment sample, while not presented in the control. Although the acquired results did not conclusively show a successful selection of antibodies against a great variety of antigens, they proved the potential of this novel approach.

Key words: parasitic plants, phage display, single-chain antibody fragments

This study is financed by the European Union-NextGenerationEU, through the National Recovery and Resilience Plan of the Republic of Bulgaria, project No. BG-RRP-2.004-0008, and grant 80-10-96 of the Sofia University Scientific Fund.

<https://doi.org/10.7546/CRABS.2025.06.07>

Introduction. Since its first introduction to the scientific community in 1985 [1], the method of displaying peptides on the surface of filamentous phages became a powerful tool in molecular biology studies. Soon after, this new technology found application in antibody selection against specific antigens, by constructing phage libraries of single-chain variable fragments (scFv) of antibodies [2]. This technology allows an entirely in vitro procedure of antibody selection and offers an antibody repertoire, similar to or greater than circulating B-cells in the body [3]. Compared to the Griffin 1 library, offering a repertoire of 10^{10} clones, based on the naïve human scFvs [3], the more recent Tomlison I + J libraries are two fully synthetic human scFv libraries with a repertoire of $\sim 10^8$ clones each. Since its introduction and free distribution by the Medical Research Council (MRC), it has been widely used in a variety of antibody selection procedures for multiple applications and has been subjected to various improvements [4]. Although in phage display technology antibody selection relies on the process of biopanning and entirely in vitro selection and propagation in bacterial cells [3, 4], which is a huge advantage over the immunization of animals, the requirement for large quantities of purified antigen remains.

Antibodies are also essential tools in plant biology studies. They are especially useful in the study of the plant cell wall [5], and in histochemical analyses of plant growth and development. One such panel of antibodies (JIM and LM antibodies) was developed by Paul Knox at Leeds University, essentially by isolating proteoglycan and oligosaccharide antigens, followed by immunization of rodents and the hybridoma approach [6]. These antibodies were proven over the years as a preferred tool of plant scientists, including in the studies of the process of parasitism by parasitic plants [7, 8], where interactions of the cell walls of both the parasite and the host are essential for the initial stages of tissue penetration. Parasitic plants are intriguing evolutionary lineages in the angiosperms, several of which cause significant agricultural impact by restricting crop plants' growth and yield [9]. Thus, the identification of defense-related mechanisms and factors of host plant resistance to parasites is of key importance to the future strategy for control of these significant weeds.

In our previous studies we found, that several tomato (*Solanum lycopersicum* L.) cultivars are at least partially resistant to the widely distributed holoparasite *Cuscuta campestris* Yunck., as primary hosts (e.g., the first host to which the seedling of the parasite attaches) [10]. Similar results were also reported by other groups, emphasizing the significance of plant cell wall lignification in this resistance [11]. Although some progress has been made in recent years, to unravel the molecular basis of host defense response to parasitic plants, the complexity of this process requires a systemic approach towards its investigation. In this study, we offer a novel application of the phage display technology for the simultaneous selection of antibodies against defense-related proteins in tomato plants subjected to *C. campestris* infection. Although not conclusively successful, this approach proved useful and promising in similar studies.

Material and methods. Plant material and growing conditions. *Cuscuta campestris* seeds were from the laboratory collection, isolate “Telish” (NCBI accession number MZ093398.1). Tomato seeds were from a commercial source, cultivar Ideal. Tomato plants were grown in greenhouse conditions in commercial soil until reaching heights of approximately 10 cm. *Cuscuta* seeds were induced for germination by a common methodology, using chemical scarification with concentrated sulphuric acid [10], then evenly distributed near the host plants until germination and coiling. Tomato stem sections with coiled *Cuscuta* were collected at different stages of the parasite development – adhesive disc formation, searching hyphae, and aborted haustoria (visual evidence of the parasite decline), and bulked to ensure coverage of all stages of parasite-host interaction.

Protein isolation, separation and Western Blotting. Water-soluble proteins were isolated by grinding of the plant tissue (100 mg) in liquid nitrogen, followed by resuspension in Pierce Plant Total Protein Extraction Kit (Thermo Scientific™) in the native lysis buffer, centrifuged at $10\,000 \times g$ for 15 min, and the protein containing supernatant was frozen until further use. Protein concentration was determined by Pierce™ BCA Protein Assay Kits (Thermo Scientific™) according to the producer’s manual. Analytical isoelectrofocusing was performed on Flatbed IEF system Multiphor II (Cleaver Scientific) with in-house made gels with carrier ampholytes SERVALYT™ pH range 3–10 in denaturing (with urea) conditions according to the instruction manual. The second dimension, as well as one-dimensional denaturing protein gel electrophoresis (SDS PAGE) was performed using the standard protocol of LAEMMLI [12] on omniPAGE Mini Vertical Protein Electrophoresis System (Cleaver Scientific). Silver gel staining was performed according to SHEVCHENKO et al. [13]. Transfer of separated proteins to nitrocellulose membrane was performed using a semi-dry blotting system (TE70XP, Hoefer Scientific), using the standard protocol of TOWBIN et al. [14]. For Western Blotting, the membrane was blocked for 2 h in 1% (w/v) bovine serum albumin in phosphate buffered saline (pH 7.4), followed by the primary antibody (overnight incubation with the membrane) – polyclonal phage suspension after four rounds of selection in 1:500 dilution in PBS (pH 7.4), followed by 1 h incubation with secondary HRP/Anti-M13 Monoclonal Conjugate (GE Healthcare) in 1:5000 dilution. Visualization was with Sigma Fast DAB substrate tablets (Sigma-Aldrich).

Phage display. Phage display was performed with the Tomlinson I and Tomlinson J synthetic human scFvs libraries, essentially following the provided MRC protocol. Briefly, the library was multiplied in *Escherichia coli* TG1 strain, using KM13 helper phage to obtain phage suspension, bearing scFvs. Selection (panning) was performed by coating Nunc-Immuno™, Nunc™ (Thermo Fischer Scientific) with 4 mL of tomato protein extract (protein extract was diluted to achieve a total of 200 µg protein per immunotube), incubation with the phage suspension, the unbound phages were discarded, and the bound ones were eluted with trypsin-PBS solution, and used for multiplication of the collected phages in

TG1. Phage titer after each round of selection was estimated by counting plaque forming units (pfu) after serial dilutions.

The major difference in our approach was the alternating rounds of positive and negative selection (Fig. 1). First, a round of positive selection was performed against protein isolate from *Cuscuta*-infected plants, collecting bound phages against control and parasitism-induced protein antigens. The following round of negative selection was performed against protein isolates from control (non-infected) plants, and the unbound phages were collected, thus enriching the phage population with clones specific for the parasitism-induced protein antigens. A total of two rounds of positive and two rounds of negative selection were performed, and the polyclonal phage suspension of the fourth round was used for Western Blot analysis to confirm the specific selection of scFvs against *Cuscuta*-induced defense proteins in tomato.

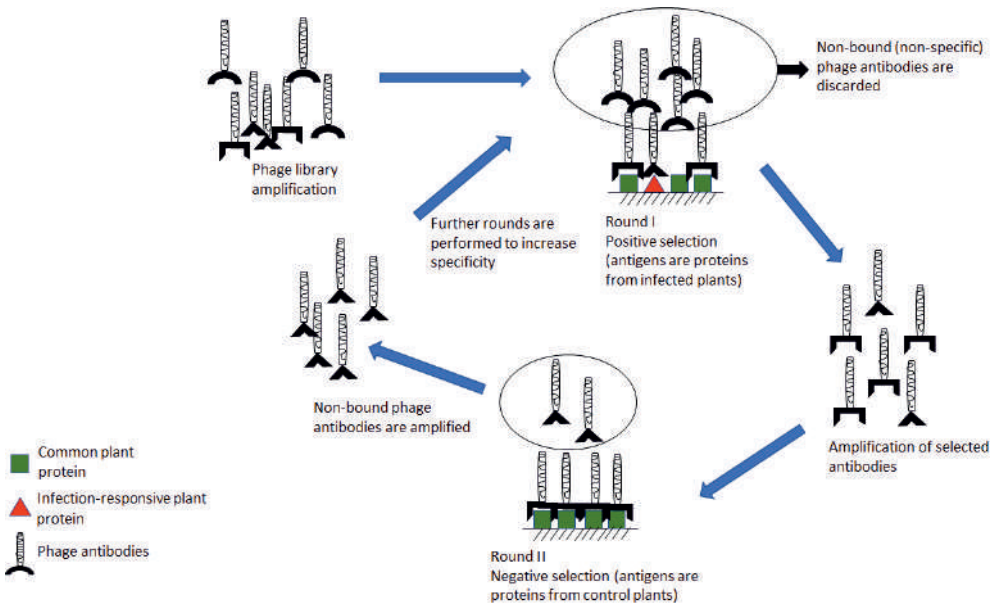


Fig. 1. Phage display for antibody selection with alternating rounds of positive and negative selection

Results and discussion. The protein profiles in the site of infection of tomato plants infected with *C. campestris* are shown in Fig. 2. Over ten differentially presented proteins were found in the infected plants, showing the proteins possibly involved in the defense reaction and resistance of the host plant. Most of them were distributed in the 40 and 25 kDa molecular weight range.

Tomato, or at least some cultivars, is known to express successful defense reactions against different *Cuscuta* species [11], and at least one specific protein receptor behind this resistance, designated CuRe1, was identified [15]. Such protein factors of resistance are of great interest, as their identification is essential in

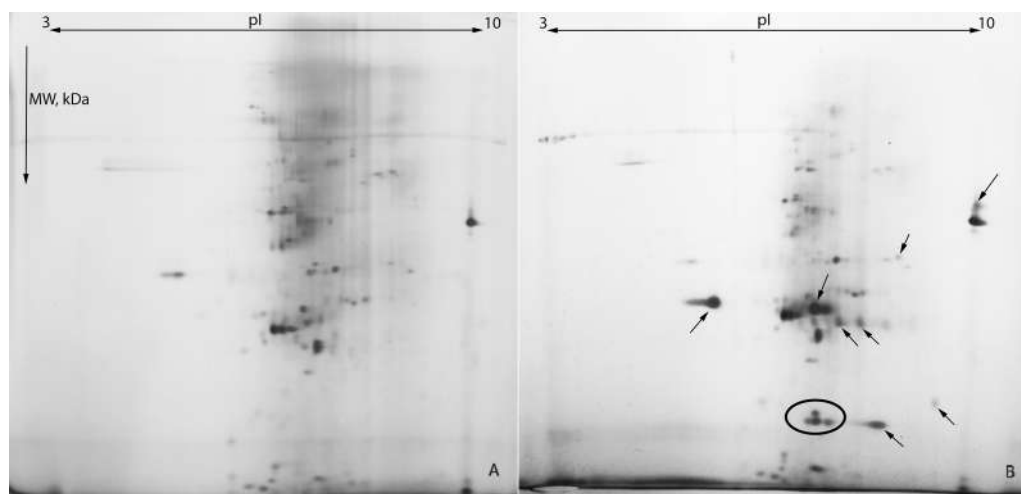


Fig. 2. Two-dimensional electrophoreses of water-soluble stem proteins from uninfected (A) and *Cuscuta campestris*-infected (B) tomato plants from the site of infection. Proteins observed only in infected plants are indicated by arrows

developing parasite-resistant crop plants [9]. However, the simple identification of differentially presented proteins in infected, compared to non-infected plants, might be misleading due to intrinsic limitations of the method (e.g., low abundance proteins might be missed, abnormal mobility of proteins, labour-intensive, etc.) [16]. The gel-free methods, on the other hand, might be hampered in this particular case by the extensive protein trafficking between parasite and host [17], which turns the interpretation of results challenging. Moreover, the proteomics identification of defense-related proteins, although of fundamental importance to understand the molecular basis of such resistance, contributes little to the fast screening of multiple cultivars. This is where specific antibodies against the desired antigen could help.

Phage display technology offers a rapid and relatively high-throughput methodology for the selection of antibodies against specific antigens. To select specific antibodies against the defense-related proteins in tomato without the purification of individual antigens, we performed antibody selection through the described phage display approach. The titers of the selected phages after each round of selection are shown in Fig. 3. As expected, the titers, acquired after the rounds of negative selection were much lower than after positive selection. However, the enrichment of phages, specific to *Cuscuta*-induced protein antigens, was nearly two orders higher after the fourth round compared to the second.

The specificity of the selected phage antibodies in polyclonal suspension was tested by Western Blot against the bulked protein isolates, initially used for the selection (Fig. 4). A single protein band was recognized, only in the *Cuscuta*-infected tomato plants, which proved at least partial success of the proposed phage

Fig. 3. Titer of selected phages (in pfu mL⁻¹) at different rounds of selection (logarithmic scale). Data in columns represent mean values $\pm$ SD ($n = 3$, technical replicates). Phage panning was performed against 200 μ g of tomato protein extract from infected plants in positive rounds (+) of selection and 200 μ g of tomato protein extract from non-infected plants in negative rounds (-) of selection

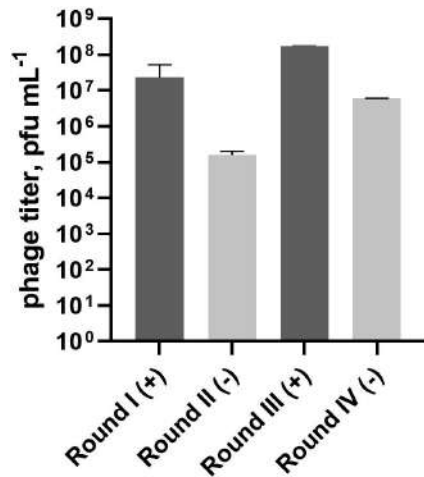


Fig. 4. Western Blot analysis of the specificity of the phage antibodies obtained from the 4th round of selection. 1. protein standards; 2. proteins from non-infected tomato; 3. proteins from *Cuscuta*-infected tomato; * recognized protein band with approximate molecular weight of 40 kDa. 12.5% T SDS PAGE, first antibody – phage displayed derived polyclonal scFv (1:500), second antibody mouse anti-M13 HRP conjugate (1:5000), development with Sigma Fast DAB tablets



display selection procedure. The protein band corresponds to 40 kDa molecular weight, where most of the differentially presented in the infected plants proteins were found (Fig. 2).

Using negative selection in phage display is not completely new, as such was employed in the so-called “Yin-Yang” panning strategy [18]. Briefly, in this approach, the phage library is subjected to one to three rounds of negative selection against common antigens (e.g., the control crude extract) in order to deplete the library of antibodies with unwanted specificity. After this, the positive selection against crude antigen solution gives higher titer and higher specificity of antibodies against the “wanted” antigens. However, the “Yin-Yang” strategy requires careful choice of the antigen mixture for the initial negative rounds of selection, involves additional steps (e.g., rounds of selection), and may lead to loss of desired antibody variety. While “next-generation phage display”, based on NGS-sequencing, intensive bioinformatics and machine learning is still cost-ineffective [19], our ap-

proach of positive/negative selection alternating rounds is offering a relatively simple alternative to classical phage display antibody selection. The specificity of the selected antibodies against a protein band in the *Cuscuta*-infected host plant, with no reactivity toward control plants, proved the efficiency of the proposed methodology. The lack of clones against multiple defense-related proteins may be due to lower titer of these specific phages, ineffective selection because of the nature of the antigens, or some of the many other reasons, hampering successful antibody selection by phage display, but still our results showed the promising potential of this approach.

Conclusions. In the present study, we offered a conceptual variety of the classical phage display technology for antibody selection, in which alternating rounds of positive and negative selection allow the acquisition of polyclonal phage suspension with displayed scFvs against multiple antigens from a crude protein extract. The main advantages of this approach are that it is relatively simple and does not require the preliminary purification of particular antigens of interest. We tested this approach in the particular case of the resistant interaction of tomato to the parasitic weed *Cuscuta campestris*, and successfully selected phage suspension reactive to the infected host plants and non-reactive to the controls. Although further studies are needed to optimize the procedure and validate the acquired phage antibodies, this study clearly shows the potential application of such an approach in a variety of studies, where rapid selection of antibodies against unidentified, response-related antigens in a crude mixture is required.

REFERENCES

- [1] SMITH G. P. (1985) Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface, *Science*, **228**(4705), 1315–1317.
- [2] MCCAFFERTY J., A. D. GRIFFITHS, G. WINTER, D. J. CHISWELL (1990) Phage antibodies: filamentous phage displaying antibody variable domains, *Nature*, **348**(6301), 552–554.
- [3] GRIFFITHS A. D., S. C. WILLIAMS, O. HARTLEY et al. (1994) Isolation of high affinity human antibodies directly from large synthetic repertoires, *EMBO J.*, **13**(14), 3245–3260.
- [4] ZHAO A., M. R. TOHIDKIA, D. L. SIEGEL et al. (2016) Phage antibody display libraries: a powerful antibody discovery platform for immunotherapy, *Crit. Rev. Biotechnol.*, **36**(2), 276–289.
- [5] WILLATS W. G., C. G. STEELE-KING, L. MCCARTNEY et al. (2000) Making and using antibody probes to study plant cell walls, *Plant Physiol. Biochem.*, **38**(1–2), 27–36.
- [6] WILLATS W. G., S. E. MARCUS, J. P. KNOX (1998) Generation of a monoclonal antibody specific to (1 → 5)- α -l-arabinan, *Carbohydrate Res.*, **308**(1–2), 149–152.
- [7] STRIBERNY B., K. KRAUSE (2015) Cell wall glycoproteins at interaction sites between parasitic giant dodder (*Cuscuta reflexa*) and its host *Pelargonium zonale*, *Plant Signal. Behav.*, **10**(11), e1086858.

- [8] LOSNER-GOSHEN D., V. H. PORTNOY, A. M. MAYER, D. M. JOEL (1998) Pectolytic activity by the haustorium of the parasitic plant *Orobanchaceae* in host roots, *Ann. Bot.*, **81**(2), 319–326.
- [9] ALBANOVA I. A., L. I. ZAGORCHEV, D. R. TEOFANOVA et al. (2023) Host resistance to parasitic plants – current knowledge and future perspectives, *Plants*, **12**(7), 1447.
- [10] ZAGORCHEV L. I., V. P. PETROVA, I. ALBANOVA et al. (2022) Salinity modulates crop plants suitability as hosts for *Cuscuta campestris* parasitism, *J. Saudi Soc. Agric. Sci.*, **21**(5), 324–330.
- [11] JHU M.-Y., M. FARHI, L. WANG et al. (2022) Heinz-resistant tomato cultivars exhibit a lignin-based resistance to field dodder (*Cuscuta campestris*) parasitism, *Plant Physiol.*, **189**(1), 129–151.
- [12] LAEMMLI U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature*, **227**(5259), 680–685.
- [13] SHEVCHENKO A., M. WILM, O. VORM, M. MANN (1996) Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels, *Anal. Chem.*, **68**(5), 850–858.
- [14] TOWBIN H., T. STAEGELIN, J. GORDON (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications, *Proc. Natl. Acad. Sci. USA*, **76**(9), 4350–4354.
- [15] FÜRST U., V. HEGENAUER, B. KAISER et al. (2016) Parasitic *Cuscuta* factor(s) and the detection by tomato initiates plant defense, *Commun. Integr. Biol.*, **9**(6), 478–481.
- [16] CHEVALIER F. (2010) Highlights on the capacities of “Gel-based” proteomics, *Proteome Science*, **8**(1), 23.
- [17] LIU N., G. SHEN, Y. XU et al. (2020) Extensive inter-plant protein transfer between *Cuscuta* parasites and their host plants, *Mol. Plant*, **13**(4), 573–585.
- [18] LIM C. C., P. C. WOO, T. S. LIM (2019) Development of a phage display panning strategy utilizing crude antigens: Isolation of MERS-CoV nucleoprotein human antibodies, *Sci. Rep.*, **9**(1), 6088.
- [19] ZAMBRANO N., G. FROEHLICH, D. LAZAREVIC et al. (2022) High-throughput monoclonal antibody discovery from phage libraries: challenging the current pre-clinical pipeline to keep the pace with the increasing mAb demand, *Cancers*, **14**(5), 1325.

Department of Biochemistry, Faculty of Biology, Sofia University “St. Kliment Ohridski”,
 8 Dragan Tsankov Blvd, 1164 Sofia, Bulgaria
 e-mails: albanova.ivanela@gmail.com, lzagorchev@biofac.uni-sofia.bg,
 modjakova@biofac.uni-sofia.bg, mkirilovm@uni-sofia.bg,
 teofanova@biofac.uni-sofia.bg