VIROLOGY 214, 235–238 (1995)

SHORT COMMUNICATION

Cucumber Mosaic Cucumovirus Antibodies from a Synthetic Phage Display Library

A. ZIEGLER,[1] L. TORRANCE, S. M. MACINTOSH, G. H. COWAN, and M. A. MAYO

Virology Department, Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA, United Kingdom

Received June 22, 1995; accepted September 19, 1995

Antibody fragments (scFv) that bind specifically to particles of cucumber mosaic cucumovirus (CMV) were obtained from a library which encodes a diverse array of synthetic antibody fragments, each displayed on the surface of filamentous bacteriophage. After four rounds of selection and enrichment, several clones were obtained which produced scFv that bound specifically to purified particles of CMV in ELISA. *Bst*NI digestion of phagemid DNA resulted in the same restriction pattern for all clones. The nucleotide sequences of three of the clones showed that they belonged to the human V_H1 family and that they had a complementarity determining region loop of 7 amino acids. Phage-displayed antibodies and soluble scFv secreted by these clones reacted with particles of CMV in sap from infected plants in ELISA. In immunoblotting tests, soluble scFv preparations reacted with SDS-denatured coat protein extracted from purified preparations of CMV isolates belonging to either subgroup I or II and also with protein extracted by SDS treatment of seeds harvested from naturally infected lupin plants. The results demonstrate the feasibility, and potential applicability, of recombinant antibody methods in plant pathology.

The use of polyclonal and monoclonal antibodies in plant virology has provided useful knowledge and insight into the processes of infection in plants, and of transmission by vectors, as well as becoming the cornerstone of the detection and diagnosis of virus diseases (*1*). Recently, it was shown that functional fragments of antibody molecules can be expressed, fused to the viral coat proteins, on the surface of filamentous phage (phage display; *2, 3*). These fragments comprise the heavy and light chain variable (antigen binding) domains of the antibody molecules linked by 15 amino acids to form a single polypeptide chain (scFv). Specific scFv can be obtained from a population of phages carrying many different scFv by binding to and then elution from antigen. The selected phage preparation can then be enriched for binders by reinfecting *Escherichia coli* with the eluted phage and repeating the procedure. In this way genetically pure populations of phage which encode the scFv can be obtained after several repeated cycles. Large combinatorial phage display libraries have been produced containing $>10^8$ different clones, and it has been shown that a range of recombinant immune reagents with diverse specificities can be isolated from such libraries (*4*).

Selection from phage display libraries yields specific antibody fragments without the need either to immunize animals or to use hybridoma technology and should, therefore, overcome several difficulties in the production of conventional polyclonal sera or monoclonal antibodies such as poor immunogenicity, toxicity of the antigens, or high production costs. However, it is not clear whether such reagents would be of use for plant virus detection and diagnosis. We have addressed this question by using as antigen cucumber mosaic cucumovirus (CMV), which is known to be a poor immunogen for the preparation of antisera by conventional means (*5*). In this paper we describe the production of scFv specific for CMV from a synthetic phage display library and their application, without further modification, to the detection of CMV in naturally infected plant tissues, in particular in seed from naturally infected lupin plants.

The scFv were obtained from the MRC human synthetic scFv library (*6*). The library of $>10^8$ clones was constructed *in vitro* using polymerase chain reactions (PCRs) to amplify the variable regions (V_H and V_L) of human immunoglobulin genes. The V_H and V_L domains contain three complementarity determining regions (CDRs) of hypervariable sequence, and the variation in these regions is largely responsible for the specificity of binding to antigen (*6*). PCRs were devised to produce DNA encoding 50 germline V_H gene segments (including CDRs 1 and 2) combined with CDR 3s of between 4 and 12 amino acids in length encoded by random nucleotide sequences (*7*). The recombination of gene segments thereby mimicked the rearrangement of immunoglobulin V_H genes that occurs *in vivo.* These rearranged V_H genes were then combined with a single $V_\lambda3$ chain in the phagemid pHEN. The scFv are fused to the minor fd phage coat protein pIII, an amber mutation between the

[1] To whom reprint requests should be addressed.

0042-6822/95 $12.00

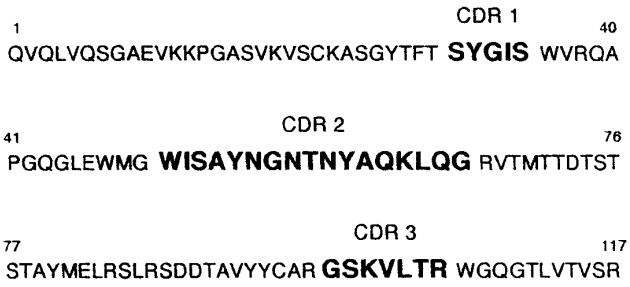

FIG. 1. Deduced amino acid sequence of the heavy chain of CMV-specific scFv. Complementarity determining regions (CDR) are shown in boldface.

sequences encoding the scFv and pIII means that strains of *E. coli* that do not suppress the amber stop codon (nonsuppressor strains) secrete soluble scFv (*9*). The scFv have a 14-residue peptide (myc-tag) attached to the C terminus which provides a means of detection and purification of soluble scFv using myc-tag-specific monoclonal antibody 9E10 (*8*).

The library stock was amplified and particles were rescued by superinfection with helper phage M13VCS, which supplies the viral genes to permit packaging of the phagemid DNA (*10*) and the display of the scFv–pIII fusion proteins on phage (*11*). For each round of selection, immunotubes (Nunc) were coated by incubation with a purified preparation of CMV (isolate R from France) at 50 μg/ml in 50 m*M* carbonate (coating) buffer, pH 9.6, overnight at 4°. The tubes were then rinsed with phosphate-buffered saline (PBS) and blocked with 2% nonfat dried milk (Marvel) in PBS. Phage preparations containing 10^{12} CFU were added to the tubes and incubated for 4 hr at room temperature (ca. 22°). After extensive washing, by rinsing the tubes 20 times with PBS containing 0.05% Tween 20 and 20 times with PBS, bound phage was eluted with 100 m*M* trimethylamine and then used to infect *E. coli* cells. Phage particles were rescued by superinfection with helper phage.

After the fourth round of selection, phage were rescued from single colonies of *E. coli* TG-1 by superinfection with the helper phage M13VCS. The resulting phage particles were collected by PEG precipitation from the culture fluids and tested by ELISA. To assay binding to CMV, microtiter plates were coated with CMV (5 μg/ml in coating buffer) and incubated with test solutions and then with polyclonal anti-M13 alkaline phosphatase conjugate. Bound alkaline phosphatase was assayed by its reaction with *p*-nitrophenyl phosphate (Sigma Immunochemicals). The polyclonal antiserum to M13VCS was produced by immunization of a New Zealand white rabbit (8×10^{12} PFU/ml M13VCS in incomplete Freund's adjuvant injected subcutaneously). Immunglobulin was precipitated from the rabbit antiserum and conjugated to alkaline phosphatase using glutaraldehyde. Twenty-four single colonies were tested and nine gave absorbance values (A_{405nm}) greater than four times the background control values (mean $A_{405nm} = 0.38$).

The DNA encoding scFv from each of these nine clones gave the same pattern of DNA fragments after digestion with *Bst*NI (*11*), suggesting that all of the clones were similar. DNA encoding the heavy chain of three clones were further compared by sequencing single-stranded DNA prepared from phage particles using primer pHEN seq (5′ CTA TGC GGC CCC ATT CA 3′) and a DNA sequencing kit (USB). The sequences were analysed using the Sequence Analysis Workshop (SAW) program (*12*). The sequences of the three clones were identical and belonged to the human V_H1 family, which is well represented in humans (*13*). The sequences of the CDR1 and CDR2 are the same as the germline gene DP-14 (*13*). The library was designed to encode CDR3 lengths of 4 to 12 amino acids, and the CDR3 of each of the clones sequenced was 7 amino acids long (Fig. 1).

Phage-displayed antibody and soluble scFv were used to detect CMV in purified preparations or in extracts of infected plants using different immunochemical techniques. Soluble scFv were produced by inducing cultures of infected HB2151 (nonsupressor strain) bacteria overnight at 25° with 1 m*M* IPTG (*9*). Periplasmic extracts containing scFv were made from cell pellets by osmotic shock, in addition the bacterial culture supernatants were filtered through a 0.45-μm filter.

The phage-displayed antibody and the preparations of soluble scFv obtained either as periplasmic extracts or as culture supernatants were tested in PTA–ELISA (*14*). Microtiter plates containing extracts of CMV strain Fny (subgroup I)-infected *Nicotiana clevelandii* and *N. glutinosa* plants (sap dilution 0.1 g/ml in coating buffer) were incubated 16 hr at 4° and then rinsed and blocked as above. The scFv preparations were mixed 1:10 with monoclonal antibody 9E10 and incubated for 3 hr at room temperature. A CMV strain LW (subgroupII) polyclonal immunoglobulin preparation at 1 μg/ml was included in the test for comparison. The plates were rinsed and incubated with anti-mouse (or anti-rabbit) alkaline phosphatase conjugate (Sigma Immunochemicals) as appropriate

TABLE 1

Absorbance Values (A_{405nm}) Obtained in PTA–ELISA of Cucumber Mosaic Virus (CMV), Tomato Aspermy Virus (TAV), and Tobacco Necrosis Virus (TNV) Isolates Using scFv Recombinant Antibody

scFv[a] (PE dilution)	Virus isolate			Noninfected plant extract
	CMV-Fny	TAV	TNV	
1:2	0.43[b]	0.06	0.09	0.09
1:5	0.21	0.13	0.1	0.08
1:10	0.15	0.08	0.1	0.07

[a] scFv preparation was a bacterial periplasmic extract (PE).
[b] Absorbance value (A_{405nm}).

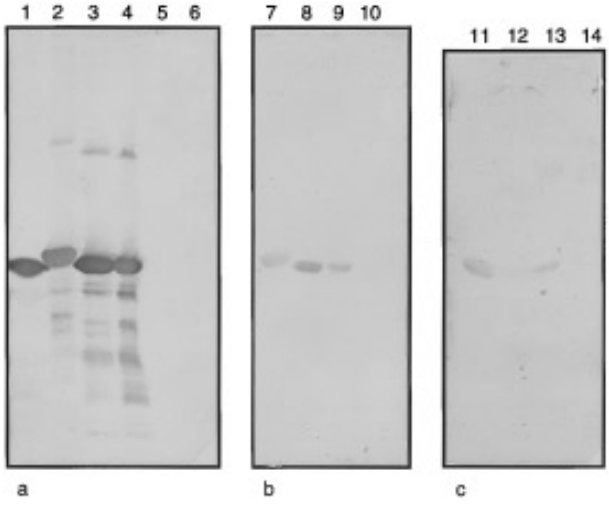

FIG. 2. Protein blots of purified preparations of CMV strains or extracts of lupin seeds (a) Probed with CMV-specific polyclonal antiserum. Lane 1, extract of infected lupin seed; lanes 2, 3, and 4, preparations of CMV strains Fny, LS, and LW, respectively; lane 5, noninfected seed; lane 6, TRV-PPK20. (b and c) Probed with CMV-specific scFv. Lanes 7, 8, and 9, preparations of CMV strains Fny, LS, and LW, respectively; lane 10, TRV-PPK20; lane 11, CMV-LW; lanes 12 and 13, infected seed extracts; lane 14, uninfected seed. For the blot in c, dormant lupin seed was soaked in water for 24 hr, then crushed and diluted 1:5 with sample buffer, and centrifuged for 5 min at 5000 *g* before 15 μl of the extract was loaded on the gels. The blot was probed with scFv and the 9E10 antibody for 4 hr before incubation with an anti-mouse alkaline phosphatase conjugate. Coomassie staining showed a single band for the purified CMV preparations. The additional bands detected by probing with CMV-specific polyclonal antiserum are probably degradation products or dimers (in the case of the bands with the higher molecular weight).

followed by substrate. The scFv was specific for CMV-Fny; it did not react with plant extracts containing tomato aspermy virus (TAV) or tobacco necrosis virus (TNV) (Table 1). The polyclonal anti-CMV-LW immunoglobulin reacted weakly with TAV giving an A_{405nm} value of 0.4 above background in the ELISA where the homologous value was 1.8.

The ability of soluble scFv to detect CMV was also tested by immunoblotting (*13*). Two micrograms of purified antigens (tobacco rattle virus coat protein from strain PPK20 was used as a negative control) was electrophoresed on a 12.5% acrylamide gel (*15*) and subsequently blotted onto nitrocellulose (*16*). After blocking in 5% nonfat dried milk the blots were incubated for 4 hr at room temperature with a mixture of the scFv and mouse monoclonal antibody 9E10. The blots were washed and incubated in anti-mouse alkaline phosphatase conjugate followed by substrate.

The scFv reacted with SDS-denatured coat protein of CMV strains Fny, LW, and LS (subgroup II) in immunoblots (Fig. 2) and also with CMV coat protein in naturally infected lupin seed extracts (Fig. 2). There was no reaction with extracts from noninfected seeds or the TRV preparation.

The scFv was shown to react with CMV by immunoblotting and PTA–ELISA. In both tests the coat protein was denatured to some extent. The scFv therefore probably bind to a continuous epitope. This is not unexpected because the phage particles carrying the scFv were selected by binding to immunotubes which had been coated with CMV antigen diluted in carbonate buffer, pH 9.6, a procedure that probably disrupts CMV particles destroying discontinuous epitopes.

Although the signal obtained in the immunoblots and ELISA was weaker than that obtained with polyclonal antiserum, the scFv were obtained after only four rounds of selection from a human synthetic phage display library and were used without any further modification. Monovalent scFv obtained from such phage libraries are known to have good to moderate binding affinities and several strategies have been suggested to improve functional affinity such as formation of multimers (*6*). Thus, shortening the linker between the V_H and V_L chains to enhance the formation of bivalent molecules, which has been shown to improve the avidity of scFv, is a plausible approach to improving the reactivity of our scFv (*17, 18*). Other strategies for improving affinity would include chain shuffling (*19*) to combine the selected heavy chain with a repertoire of light chains. Also, new larger synthetic repertoires have been constructed recently (*20*), which should enhance the prospect of selecting scFv with higher affinities.

The results reported here show that virus-specific scFv were obtained quickly (after only four rounds of selection), and without recourse to animal immunizations, from a synthetic phage display library. The results demonstrate the feasibility of using recombinant antibody methods in plant virology and, with further modification to improve avidity, we believe that such recombinant reagents will play an important role in diagnosis and in basic research on plant viruses in the future.

ACKNOWLEDGMENTS

We thank Greg Winter and Jackie Harrison, Centre for Protein Engineering, MRC, Cambridge, for supplying the phage display library; Roger Jones, Division of Plant Industries, Perth, Western Australia, for providing the LW isolate and seed collected from infected narrow-leafed lupin plants; Peter Palukaitis, Cornell University, New York, for the CMV strains Fny and LS; B. D. Harrison, Dundee University for CMV strains R and tomato aspermy virus; Stuart MacFarlane, SCRI for the TRV-PPK20 preparation; and Gottfried Himmler, Institute of Applied Microbiology, Vienna, Austria, for the 9E10 antibody. Financial support was from the Scottish Office Agriculture and Fisheries Department Flexible Fund.

REFERENCES

1. Torrance, L., *Eur. J. Plant Pathol.* 101, 351–363 (1995).
2. McCafferty, J., Griffiths, A. D., Winter, G., and Chiswell, D. J., *Nature* 348, 552–554 (1990).
3. Hoogenboom, H. R., and Winter, G., *J. Mol. Biol.* 227, 381–388 (1992).

4. Winter, G., Griffiths, A. D., Hawkins, R. E., and Hoogenboom, H. R., *Annu. Rev. Immunol.* 12, 433–455 (1994).
5. Palukaitis, P., Roossinck, M. J., Dietzgen, R. G., and Francki, R. I. B., *Adv. Virus Res.* 41, 281–348 (1992).
6. Kabat, E. A., Wu, T. T., Reid-Miller, M., Perry, H. M., and Gottesman, K. S. "Sequences of Proteins of Immunological Interest," 4th ed., pp. vii–804. U.S. Department of Health and Human Services, Bethesda, MD (1987).
7. Nissim, A., Hoogenboom, H. R., Tomlinson, I. M., Flynn, G., Midgley, C., Lane, D., and Winter, G., *EMBO J.* 13, 692–698 (1994).
8. Munro, S., and Pelham, H. R. B., *Cell* 46, 291–300 (1986).
9. Hoogenboom, H. R., Griffiths, A. D., Johnson, K. S., Chiswell, D. J., Hudson, P., and Winter, G., *Nucleic Acids Res.* 19, 4133–4137 (1991).
10. Vieira, J., and Messing, J., *Methods Enzymol.* 153, 3–11 (1987).
11. Marks, J. D., Hoogenboom, H. R., Bonnert, T. P., MacCafferty, J., Griffiths, A. D., and Winter, G., *J. Mol. Biol.* 222, 581–597 (1991).
12. Elgavish, R. A., and Schroeder, H. W., *BioTechniques* 15, 1066–1071 (1993).
13. Tomlinson, I. M., Walter, G., Marks, J. D., Llewelyn, M. B., and Winter, G., *J. Mol. Biol.* 227, 776–798 (1992).
14. Torrance, L., *In* Techniques for the Rapid Detection of Plant Pathogens (J. M. Duncan and L. Torrance, Eds.). Blackwell, Oxford, 1992.
15. Laemmli, U. K., *Nature* 227, 680–685 (1970).
16. Towbin, H., Staehelin, T., and Gordin, J., *Proc. Natl. Acad. Sci. USA* 76, 4350 (1979).
17. Holliger, P., Prospero, T. D., and Winter, G., *Proc. Natl. Acad. Sci. USA* 90, 6444–6448 (1993).
18. Pack, P., Kujau, M., Schroeckh, V., Knüpfer, U., Wenderoth, R., Riesenberg, D., Plückthun. A., *BioTechnology* 11, 1271–1276 (1993).
19. Clackson, T., Hoogenboom, H. R., Griffiths, A. D., and Winter, G., *Nature* 352, 624–628 (1991).
20. Griffiths, A. D., Williams, S. C., Hartley, O., Tomlinson, I. M., Waterhouse, P., Crosby, W. L., Kontermann, R. E., Jones, P. T., Low, N. M., Allison, T. J., Prospero, T. D., Hoogenboom, H. R., Nissim, A., Cox, J. P. L., Harrison, J. L., Zaccolo, M., Gherardi, E., and Winter, G., *EMBO J.* 13, 3245–3260 (1994).