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EXPERIMENTAL ARTICLES

Isolation, Purification, and Identification of the Virulence Protein VirE2 from *Agrobacterium tumefaciens*

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Abstract—Bacteria of the genus *Agrobacterium* are capable of transferring a fragment of their Ti-plasmid T-DNA, in a complex with the proteins VirE2 and VirD2, into the nuclei of plant cells and incorporating it into the chromosome of the host. The mechanisms of T-DNA transportation through the membrane and cytoplasm of the plant cell are unknown. The aim of this work was isolation of the virulence protein VirE2 for studying its role in T-DNA transportation through the membrane and cytoplasm of eukaryotic cells. For VirE2 accumulation, the *virE2* gene was cloned into plasmid pQE31. VirE2 was isolated from the cells of *E. coli* strain XL1-blue, containing the recombinant plasmid pQE31-*virE2*. The cells were disrupted ultrasonically, and the protein, with six histidine residues at the N-end, was isolated by means of affinity chromatography on a Ni-NTA-superose column. The purified protein was tested by the immunodot method using polyclonal rabbit antibodies and anti-VirE2 miniantibodies. The ability of the recombinant protein VirE2 to bind to single-stranded DNA was judged from the formation of complexes detected by electrophoresis in agarose gel. Thus, we isolated, purified, and partially characterized the *Agrobacterium tumefaciens* virulence protein VirE2, which is capable of binding to single-stranded T-DNA upon transfer to the plant cell.

Key words: Agrobacterium tumefaciens, virulence protein VirE2, cloning in *E. coli*, affinity chromatography, phage display, miniantibodies.

The soil bacterium *Agrobacterium* causes formation of undifferentiated tumors in plants via transfer of agrobacterial T-DNA, which carries genes regulating phytohormone synthesis, into a plant genome [1]. In the natural environment, expression of virulence genes controlling T-DNA transfer is triggered by the phenolic compounds acetosyringone and hydroxyacetosyringone, the precursors in the cell wall synthesis, upon damage of the plant. After T-DNA incorporation, tumor cells start production of specific compounds that serve as the sole source of carbon and nitrogen for agrobacteria. The specificity of these substrates provides agrobacteria with extra benefits in a new ecological niche. Any genes located between the left and right T-DNA borders can be transferred. Expression of artificially introduced genes in a host cell modifies its properties, which has been extensively used in agricultural biotechnology for creation of transgenic plants.

T-DNA was shown to be transferred in the form of a DNA–protein complex (T-complex). The T-complex comprises T-DNA, VirD2 protein attached to the 5'-end of the DNA strand, and VirE2 protein covering the whole DNA strand (600 molecules). The complex T-DNA–VirD2 and the VirE2 protein are transferred to plant cells independently. VirE2 export requires the presence of VirE1 protein in the cell [2, 3], and the VirE2/VirE1 complex is transferred through a VirB-independent channel [4]. VirE2 export can be selectively blocked by introduction of plasmid pSa into agrobacteria. This plasmid encodes the protein pilin, which is assumed to block VirE2 efflux from cells [5]. It was found that VirE2 protein reacts with a flat lipid membrane and forms a membrane pore that opens under exposure to an electric field with an intensity of 100 mV and can serve as a passageway for short oligonucleotides [6]. However, VirE2-related mechanisms of T-DNA transfer through artificial and natural membranes are not known.

This work was aimed at isolation of the protein VirE2 and generation of anti-VirE2 miniantibodies.

MATERIALS AND METHODS

Reagents. The following reagents were used throughout this study: polyacrylamide, bis-acrylamide, TEMED, ammonium persulfate, DTT (Carl Roth, Germany), agarose, PMSF (phenylmethylsulfonyl fluoride), IPTG (isopropyl β-D-thiogalactopyranoside), SiO_2, Tris–HCl, EDTA (Sigma, United States), NaCl, KCl, $CaCl_2$, $MgCl_2$, NaH_2PO_4, NaOH, sodium tetrabo-

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rate (Russia), Coomassie Brilliant Blue R250 (Sigma), BSA (bovine serum albumin) (Serva, United States), *E. coli* SsB-protein (single strand binding protein) (Promega, United States), imidazole, Tween 20 (Fluka, Germany), Ni-NTA-superose (Amersham Pharmacia Biotech, United Kingdom), peptone, tryptone, yeast extract, glycine–HCl (Dia-M, Russia), PEG 6000 (Ferak, Germany), DAB (3'3-diaminobenzidine) (Sigma), sepharose CL-4B (Amersham Pharmacia Biotech), restriction endonucleases (*Bam*H1, *Sac*1, *Eco*RI, and *Hin*dIII), *Taq* polymerase (MBI Fermentas, Lithuania), *Aspergillus oryzae* S1 nuclease, the corresponding buffers (Promega), PCR primers (Sintol, Russia), and DNA molecular markers (Promega).

Protein VirE2 was tested with scFv antibodies and monoclonal mouse anti-c-Myc antibodies (Sigma). Also, horseradish peroxidase–conjugated antimouse antibodies were used (Amersham Pharmacia Biotech). Polyclonal VirE2-specific antibodies, obtained by immunization of rabbits with a preparation of protein VirE2 from *A. tumefaciens*, were kindly provided by C. Baron (Germany).

The following antibiotics were used in this study: ampicillin (Ap), 50–100 μg/ml; rifampicin (Rif), 50–100 μg/ml; tetracycline (Tc), 10 μg/ml; and kanamycin (Km), 50–100 μg/ml.

Strains, plasmids, and media. The strains *Escherichia coli* TG1 SupE Δ (hsd M mcr B) 5 ($rk^- mk^-$ mcr B^-) thi Δ (*lac-pro* AB) [F' *tra* D36 *lac* I^q *Lac* Z M15] and *Escherichia coli* JS5 Ara D 39 Δ (ara leu) 7967 Δ (*lac*)x74 gal U galKhsdR12 ($rk^- mk^-$) mcr A mcr BC rpsL (str^r) thi rec A1 [F::Tn10 (tet^r)proAB lac I^q Lac Z M15], as well as the library of scFv antibodies, were kindly provided by A. Laman (the Branch of Shemyakin–Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences). The strains *Agrobacterium tumefaciens* C58 and *Escherichia coli* XL1-Blue (F' $proAB^{lacIq}D$(lacZ)M15 Tn10(Tetr)/recA1 endA1 gyrA96(Nal^r) thi1 hsdR17(rk^- mk+) supE44 relA1 lac) and plasmid pBI101.1, containing T-DNA of the *A. tumefaciens* Ti-plasmid [7], were obtained from the collection of the Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences (IBPPM RAS). Plasmid pQE31 was purchased from Promega. Phage λ was purchased from MBI Fermentas. *E. coli* was cultivated on 2× YT medium. *A. tumefaciens* was cultivated on YT medium.

Isolation of plasmid DNA from *E. coli* cells was carried out on Promega columns in accordance with the manufacturer's protocols.

Isolation of genomic DNA from bacterial cells was carried out according to the standard method [8].

Electrophoresis of DNA and protein preparations was carried out in a 0.8% agarose gel using Tris–acetate buffer. Plasmid DNA was digested with endonucleases *Eco*RI and *Hin*dIII (Promega, MBI Fermentas) taken in two- to threefold excess according to the manufacturer's instructions. Gels were stained with ethidium bromide (0.5 μg/ml). Molecular weight markers were purchased from Promega. Vertical electrophoresis of protein preparations was carried out under denaturing conditions in 12.5% SDS-PAAG according to [9].

Total protein content was determined by the Bradford method [10]. BSA (Serva) was used for calibration.

Cloning of *virE2* gene. The *vir*E2 gene was cloned by PCR using the vector pQE31. Total *Agrobacterium tumefaciens* C58 DNA served as the matrix. The following PCR primers complementary to the left and right *vir*E2 borders (Sintol) were used: VirE2 (+), 5'-GAC-GAT-GGA-TCC-GAA-GGG-CGA-AG-3'; VirE2(–), 5'-AAAA-CTG-CAG-CTA-CAG-ACT-GTT-TAC-GGT-TGG-GCC-GC-3'. Primers were annealed at 62°C. The temperature regime was as follows: initial incubation at 95°C for 5 min; 35 cycles at 94°C, 45 s, 62°C, 45 s, 72 °C, 1 min, 72°C, 10 min, and cooling at 4°C. The reaction mixture contained H_2O, 12.8 μl; dNTP, 2 mM (concentrated); PCR buffer (50 mM Tris-HCl, pH 8.6; 50 mM KCl; 2.5 mM $MgCl_2$; Tween 20, 1%), 2 μl; *Tag* polymerase, 1 μl; *A. tumefaciens* C58 DNA (1 mg/ml), 1 μl; 20 pM primer VirE2(+), 1 μl; 20 pM primer VirE2(–), 1 μl. The PCR product was isolated from agarose gel and cloned into vector pQE31 at the sites *Bam*HI and *Pst*I. Preparation of competent cells and *E. coli* transformation was carried out according to [11].

Isolation and purification of VirE2 protein. The recombinant VirE2 protein with six histidine residues at the N-end was isolated by affinity chromatography on an Ni-NTA-superose column as recommended by the manufacturer. *E. coli* strain XL1-blue containing the *virE2* gene on plasmid pQE32 was grown overnight in 5 ml of 2× YT medium containing 2% glucose and the corresponding antibiotics at 37°C. The broth was diluted with fresh medium (1 : 100) and incubated for 3.5 h. Then, cells were washed with 50 mM NaCl and suspended in 1 mM IPTG for the induction of expression of the recombinant protein. The suspension was incubated for 4 h on a shaker at 28°C. Then, cells were sedimented by centrifugation and the pellet was resuspended in buffer A (50 mM NaH_2PO_4, 300 mM NaCl, 5 mM imidazole, 1 mM PMSF, pH 8.0) and subjected to sonication six times with a 30-s interval between impulses. Cell debris was sedimented by centrifugation, and the supernatant was applied on a Ni-NTA-superose column equilibrated with Buffer A with a speed of 0.5 ml/min. Nonspecifically bound proteins were washed with four volumes of buffer B (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0). VirE2 protein was eluted with buffer C (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0). Total protein isolated from untransformed *E. coli* XL1-blue cells was used as control.

Postpurification of the protein for antibody generation was carried out by two independent methods. Electrophoresis was followed by elution from PAAG tracks, dialysis, and protein reprecipitation. Then, the protein mixture was fractionated in tubes with membrane fil-

ters (Prinston Separation, United States) allowing separation of proteins with different molecular weights.

Generation of anti-VirE2 miniantibodies. Anti-VirE2 miniantibodies were generated by the phage display method [12]. We used a combinatorial mouse scFv (single-chain fragment variable) miniantibody library displayed on filamentous phages. The library was created as described in [13]. Selection of clones bearing anti-VirE2 miniantibodies in their capsid was carried out by panning the library on VirE2 protein immobilized on a plate. A suspension of phage particles representing the whole pool of the library (10^{11}–10^{12} particles/ml) was dispensed into the plate wells (100 μl per well), containing immunosorbent. The plate was incubated overnight. Then, the supernatant was removed and the plate was blocked with 3% BSA. The nonspecifically adherent phagemid particles were removed by tenfold repeated washing with PBS buffer supplemented with 0.1% Tween 20. Then, wells were thrice washed with PBS for removal of traces of detergent. Specifically bound particles were eluted by addition of 100 μl of 100 mM glycine–HCl, pH 2.5. Eluted phages were used to infect *E. coli* cells. The second and the third rounds were carried out according to the same scheme. Phagemids from selected clones were isolated as follows. A culture of *E. coli* containing the recombinant plasmid pHEN1 and M13-K07 helper phage was grown overnight and centrifuged at 8 000 g for 10 min. The supernatant was mixed with 0.2 volume of PEG/NaCl (20% PEG, 2.5 M NaCl) and incubated for 1 h at 4°C. After centrifugation, the pellet was suspended in 0.1 volume of TE buffer and once more precipitated with PEG/NaCl under the same conditions. The precipitate was resuspended in 0.01 volume of TE buffer. After *E. coli* transduction and plating onto YT agar, the titer of phagemid particles was determined.

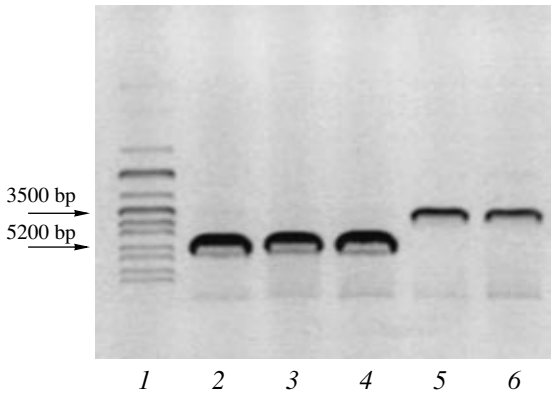

Fig. 1. Electrophoresis of DNA preparations from *E. coli* clones bearing *virE2* gene in plasmid pQE31: (*1*) molecular weight markers; (*2–4*) plasmid pQE31 with inserted *virE2* gene, hydrolyzed with *Bam*HI endonuclease; (*5*) plasmid pQE31 without inserted *virE2* gene, hydrolyzed with *Bam*HI endonuclease; (*6*) plasmid pQE31 hydrolyzed with *Bam*HI endonuclease.

For isolation of antibodies secreted by *E. coli* into periplasm and culture broth, selected phagemids were introduced into *E. coli* JS5 cells via transfection, and YT agar with ampicillin was inoculated. The obtained clones were purified by successive reincubation. Miniantibodies were accumulated in culture broth to preparation levels as follows. An overnight culture was diluted 1 : 100 and incubated until an optical density of 0.5 at 600 nm was obtained. Then, cells were sedimented by centrifugation, washed with 50 mM NaCl, resuspended in 1000 ml of a fresh medium containing 1 mM IPTG, and incubated for 3 h on a shaker at 25°C. Then, the culture was cooled on ice and centrifuged, and the pellet was resuspended in 10 ml of 200 mM sodium tetraborate, 160 mM NaCl, and 1 mM EDTA, pH 8.0, to provide efflux of miniantibodies from periplasm to the solution. The preparation was centrifuged at 4000 *g* and 4°C for 10 min, and the sediment was disposed of. Cell debris was removed by centrifugation at 12000 *g* for 30 min. We obtained 1.2–1.5 mg of a crude preparation of anti-VirE2 scFv miniantibodies from 1000 ml of culture broth. Then, the preparation was dialyzed against 300 volumes of PBS buffer and purified by affinity chromatography on sepharose CL-4B–protein A as described earlier [13].

Detection of VirE2 ability to bind to single-stranded DNA. In vitro interaction of VirE2 protein with single-stranded DNA was judged from the formation of complexes of high molecular weight after the incubation of purified VirE2 with DNA. Complexes were detected by electrophoresis in agarose gel.

Immunodot method. VirE2 was detected by immunodot and immunoblot assays using rabbit polyclonal VirE2-specific antibodies and scFv antibodies. Protein was revealed on nitrocellulose filters with a pore diameter of 1.5 μm (Millipore, United States) divided into squares of 5 × 5 mm. Antigen solution (1–2 μl) was dispensed in the center of each square, dried, and fixed at 40°C for 5 min. To prevent nonspecific adsorption, the membrane was incubated in a solution of blocking buffer (PBS, 1% BSA, 0.02% Tween 20, pH 7.2) for 2 h at room temperature. Then, the membrane was incubated in a solution of primary antibodies (rabbit polyclonal antibodies, 10 μg/ml) for 1 h at 37°C and thrice washed with PBS buffer containing 0.02% Tween 20. Then, the membrane was incubated in a solution of secondary horseradish peroxidase–conjugated antirabbit antibodies. DAB served as the substrate.

In other experiments, the membrane was incubated with anti-VirE2 scFv antibodies (10 μg/ml) for 2 h at room temperature. Then, primary antibodies were removed by washing, and the membrane was incubated with anti-c-Myc antibodies for 2 h at room temperature. Then, the membrane was washed, incubated with horseradish peroxidase–conjugated antimouse antibodies for 1 h at 37°C, washed once more, and visualized histochemically using DAB. For direct labeling, colloidal gold–conjugated scFv antibodies of 15 nm in diameter

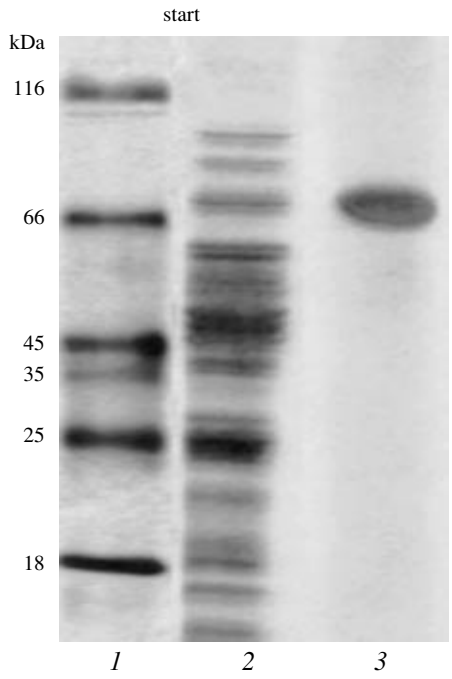

Fig. 2. Denaturing electrophoresis in 12.5% PAAG of extracts containing protein VirE2 (staining with $AgNO_3$): (*1*) molecular weight markers (kDa); (*2*) crude cell extract of IPTG-induced *E. coli* XL1-blue cells bearing *virE2* gene inserted in plasmid pQE31; (*3*) protein VirE2 purified by affinity chromatography.

were obtained. These were used for VirE2 visualization on nitrocellulose membranes as described earlier [14].

RESULTS AND DISCUSSION

Cloning of the gene encoding VirE2 protein. The *virE2* gene was cloned by PCR. Total *Agrobacterium tumefaciens* C58 DNA served as the matrix for amplification. PCR primers were selected from the primary oligonucleotide sequence of the *virE2* gene (see Materials and Methods). The size of the fragment corresponding to the *virE2* gene was estimated as 1.67 kb. The obtained PCR fragment was isolated from agarose gel and cloned on plasmid pQE31 at sites *Bam*HI and *Pst*I (Fig. 1).

Preparation of purified VirE2 protein. Protein extract containing VirE2 protein with six histidine residues at the N-end was purified of *E. coli* proteins by affinity chromatography on a Ni-NTA-superose column. The results are presented in Fig. 2.

It is clearly visible that the third track contains one band corresponding to the VirE2 molecular weight (60.6 kDa) [4, 6]. VirE2 identification and analysis of its binding ability are described below.

Postpurification of VirE2 protein for antibody generation was carried out by two independent methods, electrophoresis followed by elution, dialysis, and reprecipitation from PAAG tracks, and fractionation of the protein mixture in tubes with membrane filters (Prinston Separation) allowing separation of proteins with different molecular weights.

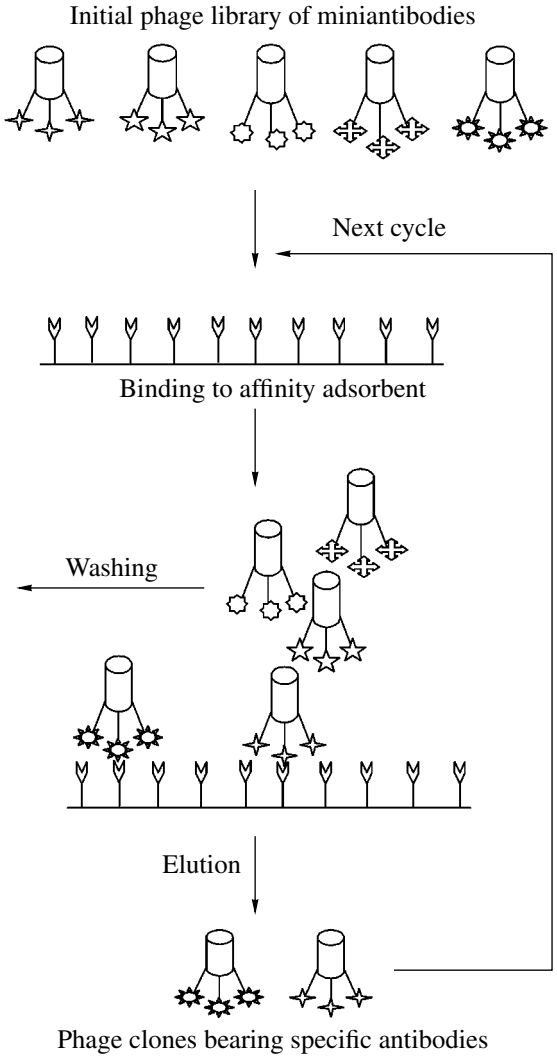

Fig. 3. Miniantibody selection by the phage display method.

Generation of marked anti-VirE2 miniantibodies. Anti-VirE2 miniantibodies were generated by the phage display method as described in Materials and Methods (Fig. 3). Anti-VirE2 miniantibodies were conjugated to colloidal gold particles. The miniantibody concentration was 10 μg/ml.

Identification of VirE2 with antibodies. Polyclonal antibodies against *A. tumefaciens* VirE2 were used to test the purified recombinant VirE2 protein and supernatant from culture broth of *E. coli* XL1-blue carrying *virE2* gene on plasmid pQE31. Figure 4a presents a positive reaction of polyclonal antibodies with VirE2 protein (squares *1, 2*), a negative control, and a negative reaction in the fraction of proteins that did not bind to Ni-NTA-superose (squares *3, 4*). VirE2 protein isolated from IPTG-induced *E. coli* XL1-blue cells gave a posi-

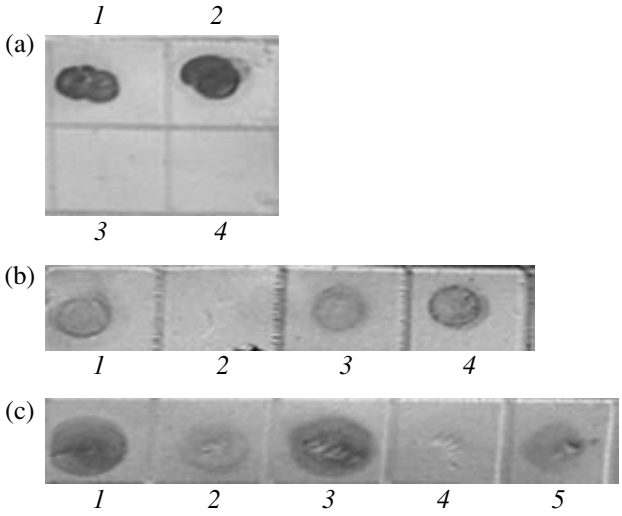

Fig. 4. Immunoenzyme dot assay of protein VirE2 using (a) rabbit polyclonal anti-VirE2 antibodies, (b) scFv anti-VirE2 antibodies, and (c) colloidal gold–conjugated scFv anti-VirE2 antibodies: (a*1*, b*3*, c*1*) crude cell extract of IPTG-induced *E. coli* XL1-blue cells bearing *virE2* gene in plasmid pQE31; (a*2*, b*4*, c*3*) chromatographically purified protein VirE2; (a*3*, b*2*, c*4*) crude cell extract of *E. coli* XL1-blue cells; (a*4*) fraction of proteins that did not bind to Ni-NTA-superose; (b*1*, c*5*) crude extract of *A. tumefaciens* C58 cells after induction of *vir* genes with acetosyringone; (c*2*) crude extract of non-IPTG-induced *E. coli* XL1-blue cells bearing *virE2* gene in plasmid pQE31.

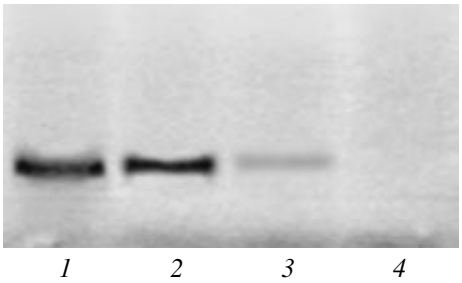

Fig. 5. Comparison of the abilities of protein VirE2 and *E. coli* Ssb-protein to react with single-stranded DNA: (*1*) single-stranded phage λ DNA without S1 nuclease; (*2*) single-stranded phage λ DNA + S1 nuclease + VirE2-containing extract; (*3*) single-stranded phage λ DNA + S1 nuclease + *E. coli* Ssb-protein; (*4*) single-stranded phage λ DNA + S1 nuclease.

tive reaction with scFv antibodies against *A. tumefaciens* VirE2 (Fig. 4b). In addition, VirE2 protein was tested with colloidal gold–conjugated scFv antibodies (Fig. 4c).

The presented pictures show that the recombinant VirE2 protein and *A. tumefaciens* VirE2 protein are equally detected with the generated antibodies.

Analysis of VirE2 binding and protective abilities. The assembled structure of the T-complex in vitro is rigid, which prevents globulation of T-DNA [15, 16]. The necessity of transportation of nuclear acids in plant cells in an uncoiled form was postulated for viral RNA [17, 18]. It is known that approximately 600 molecules of VirE2 protein are required to cover one strand of T-DNA [19]. VirE2 protein belongs to the family of Ssb-proteins, which bind to single-stranded DNA in a nonspecific, cooperative way.

Ability of the isolated recombinant VirE2 protein to react with single-stranded DNA and to protect it from plant nuclease degradation was checked in model experiments with S1 nuclease, which hydrolyzes single-stranded DNA. Double-stranded phage λ DNA was used as the model after denaturation at 95°C for 5 min followed by quick cooling in a water bath with ice. The solution of denatured phage λ DNA was mixed with the solution of the tested VirE2 protein, and S1 nuclease was added to the mixture. *E. coli* Ssb-protein, which has a molecular weight of 19.4 kDa and is capable of binding to single-stranded DNA in a nonspecific cooperative way, was used as the control. Optimal reaction parameters, i.e., quantity of S1 nuclease, DNA concentration, and reaction time, were determined in preliminary experiments.

An analysis of the DNA–protein interaction is presented in Fig. 5. The protective ability of VirE2 protein was much more pronounced than that of *E. coli* Ssb-protein (tracks *2, 3*). Thus, the isolated recombinant VirE2 protein exhibits functions of Ssb-like protein.

Thus, *Agrobacterium tumefaciens virE2* gene was cloned into *E. coli* and a superproducer of VirE2 protein was obtained. VirE2 × 6His protein was isolated and purified by affinity chromatography, and VirE2 ability to react with single-stranded DNA was proved. Anti-VirE2 antibodies were generated. For the first time, VirE2 protein was directly labeled with colloidal gold.

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REFERENCES

1. Christie, P.J., *Agrobacterium tumefaciens* T-Complex Transport Apparatus: A Paradigm for a New Family of

Multifunctional Transporters in Eubacteria, *J. Bacteriol.*, 1997, vol. 179, pp. 3085–3094.

2. Sundberg, C., Meek, L., Carroll, K., Das, A., and Ream, W., VirE1 Protein Mediated Export of the Single-Stranded DNA Binding Protein VirE2 from *Agrobacterium tumefaciens* into Plant Cells, *J. Bacteriol.*, 1996, vol. 78, pp. 1207–1212.
3. Mysori, R.S., Bassuner, D., Deng, X.B., Darfinian, N.S., Motchoulski, A., and Ream, W., Role of *Agrobacterium tumefaciens* VirD2 Protein in T-DNA Transfer and Integration, *MPMI*, 1998, vol. 11, pp. 662–683.
4. Nhen, L., Li, C., and Nester, E., Transferred DNA (T-DNA)-associated Proteins of *Agrobacterium tumefaciens* Are Exported Independently of *virB, Proc. Natl. Acad. Sci. USA*, 2000, vol. 97, pp. 7545–7550.
5. Lee, L.-Y., Gelvin, S.B., and Kado, C.I., pSa Causes Oncogenic Suppression of *Agrobacterium* by Inhibiting VirE2 Protein Export, *J. Bacteriol.*, 1999, vol. 181, pp. 186–196.
6. Dumas, F., Duckely, M., Pelczar, P., Van Gelder, P., and Hohn, B., An *Agrobacterium* VirE2 Channel for Transferred-DNA Transport into Plant Cells, *Proc. Natl. Acad. Sci. USA*, 2001, vol. 98, pp. 485–490.
7. Jefferson, R.A., Kavanagh, T.A., and Bevan, M.W., GUS Fusions: Beta-Glucuronidase as a Sensitive and Versatile Gene Fusion Marker in Higher Plants, *EMBO J.*, 1987, vol. 20, pp. 3901–3907.
8. *Gennaya inzheneriya rastenii. Laboratornoe rukodstvo* (Genetic Engineering of Plants. A Laboratory Manual) Dreiper, Dzh., *et al.*, Eds., Moscow: Mir, 1991, pp. 76–77 (Russian Translation).
9. Laemmli, U.K., Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4, *Nature*, 1970, vol. 227, pp. 680–685.
10. Bradford, M.M., A Rapid Sensitive Method for Quantification of Microgram Quantities of Protein Utilizing the Principle of Protein–Dye Binding, *Anal. Biochem.*, 1976, vol. 72, pp. 248–254.
11. Sambrook, J., Fritsch, E.F., and Maniatis, T., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, New York: Cold Spring Harbor Lab., 1989.
12. McCafferty, J. and Johnson, K.S., Construction and Screening of Antibody Display Libraries, *Phage Display of Peptides and Proteins. A Laboratory Manual*, Kay, B.K. *et al.*, Eds., Academic, 1996, pp. 79–112.
13. Laman, A.G., Shepelyakovskaya, A.O., Ulitin, A.B., Markova, E.V., Mareeva, T.Yu., Bystrov, N.S., Brovko, F.A., and Nesmeyanov, V.A., Production of Miniantibodies against Human Granulocyte Colony–Stimulating Factor with the Use of Combinatory Library of Mouse ssFv, *Bioorg. Khim.*, 2002, vol. 28, pp. 126–134.
14. Chumakov, M.I. and Kurbanova, I.V., Localization of Protein VirB1 on the Surface of Agrobacteria, *Biol. Membr.*, 1998, vol. 15, pp. 719–721.
15. Citovsky, V., Guralnik, B., Simon, M.N., and Wall, J.S., The Molecular Structure of *Agrobacterium* VirE2–Single DNA Complexes Involved in Nuclear Import, *J. Mol. Biol.*, 1997, vol. 272, pp. 718–727.
16. Tzfira, T., Rhee, Y., Chen, M.-H., Kunik, T., and Citovsky, V., Nuclear Acid Transport in Plant–Microbe Interactions: The Molecules That Walk Through the Walls, *Annu. Rev. Microbiol.*, 2000, vol. 54, pp. 187–219.
17. Citovsky, V.C., Wong, M.L., and Zambryski, P., Cooperative Interaction of *Agrobacterium* VirE2 Protein with Single-Stranded DNA: Implications for the T-DNA Transfer Process, *Proc. Natl. Acad. Sci. USA*, 1989, vol. 86, pp. 1193–1197.
18. Howard, E. and Citovsky, V., The Emerging Structure of the *Agrobacterium* T-DNA Transfer Complex, *BioEssays*, 1990, vol. 12, pp. 103–108.
19. Rossi, L., Tinland, B., and Hohn, B., Role of Virulence Proteins of *Agrobacterium* in the Plant, *The Rhizobiaceae. Molecular Biology of Model Plant-Associated Bacteria*, Spaink, H.P. *et al.*, Eds., Dordrecht: Kluwer Academic, 1998, pp. 303–320.