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Visual Representation by Atomic Force Microscopy (AFM) of Tomato Spotted Wilt Virus Ribonucleoproteins

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Atomic force microscopy (AFM) allows the observation of biological material without fixation procedures. Here we present AFM images of ribonucleoproteins (nucleocapsids) derived from a plant infecting RNA virus (tomato spotted wilt virus, TSWV), which have been recorded in contact mode. The nucleocapsids, prepared from systemically infected leaves of tobacco, were spreaded on a glass surface and dried in air, and appeared as regularly formed rings, resembling the proposed pseudocircular and panhandle structure of encapsidated genomic RNA. Average values between 1300 and 2200 nm of nucleocapsid lengths could be related to dimensions estimated by electron microscopy, thereby validating a filamentous configuration of the TSWV ribonucleoproteins. However, to our knowledge regular, ring-like forms of ribonucleoproteins have not been obtained by electron microscopy, which rather showed an amorphous structure of the virus particles. Hence, the AFM approach provides a starting point for further detailed studies on TSWV ribonucleoprotein complexes.

Key words: Atomic force microscopy / *Bunyaviridae* / Plant virus / Ribonucleoprotein / TSWV.

Introduction

Tomato spotted wilt virus is the prototype member of the plant infecting *Tospovirus* genus in the family *Bunyaviridae*, a large group of predominantly vertebrate- and insect infecting, enveloped RNA viruses (Elliott, 1996; Goldbach and Peters, 1996; for a review see Adkins, 2000). Their genomes are composed of three single-stranded RNA species, each consisting of complementary terminal sequences which allow the formation of pseudocircular (panhandle) structures. The three genomic segments are separately encapsidated by multiple copies of the viral N protein resulting in ribonucleoprotein complexes (nucleocapsids). The shape and structure of enveloped virions of the *Bunyaviridae* was investigated in several studies (for a review see Elliott, 1996). Electron micrographs of nucleocapsids derived from Lumbo virus, a member of the human pathogenic *Bunyavirus* genus, revealed the existance of circular molecules, suggesting that the RNA inside the virion is present in a covalently closed circular form (Samso *et al.*, 1975, 1976). Negative contrast electron micrographs showed convoluted, supercoiled and apparently circular nucleocapsids from La Crosse virus (genus *Bunyavirus*) with 12 nm mean diameter and a modal length between 200 and 700 nm of the filaments (Obijeski *et al.*, 1976).

Studies on subviral particles from TSWV using electron microscopy showed ribonucleoprotein complexes resembling those of influenza virus (Mohamed, 1981). In infected plant cells, moderately dense amorphous material (viroplasm) has been identified (Ie, 1982) which mainly consists of N protein. The examination of osmium-fixed ultrathin sections further revealed that the viroplasm is comprised of small complexes loosely arranged in apparent chains or strings. These aggregates exhibited cubic, circular, or eliptic profiles with diameters of up to 120 nm, and a striated arrangement could be differentiated (Kitajima *et al.*, 1992; Goldbach and Peters, 1996). Since the complexes reacted with an antiserum against the N protein, they were believed to represent an assembly of nucleocapsids.

Our goal was to investigate the structure of TSWV ribonucleoproteins by atomic force microscopy (AFM) in order to enhance our understanding of their assembly and morphology. Applications of AFM to biological materials have remarkably increased during recent years (Kasas *et al.*, 1997), and it was shown that AFM can yield topographic images of biomolecules in various environments without complicated pretreatment of the sample.

Results

Leaves systemically infected with TSWV were harvested and ribonucleoproteins were purified. After separation of a crude virion extract obtained from sucrose cushion sedimentations followed by cesium sulfate density gradients, a homogeneous, UV-light absorbing fraction was obtained. The integrity of the ribonucleoprotein isolation was determined by SDS-polyacrylamide gel ectrophoresis, which showed that the fractions contained a distinct protein with an apparent molecular mass of 28 kDa, closely correlating with the estimated molecular mass of the TSWV nucleocapsid protein (N, 28.8 kDa; Maiss *et al.*, 1991; Figure 1A). Immunoreactivity with TSWV-specific antibodies substantiated the identity of the isolated material as TSWV nucleocapsid protein (Figure 1B).

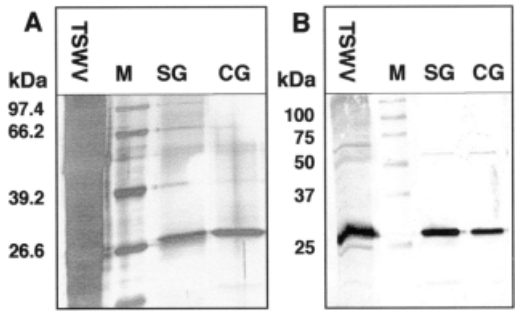

Fig. 1 Gel Electrophoresis and Western Blot Analysis.
(A) Aliquots of a crude protein extract from infected leaves (TSWV) were separated on denaturing polyacrylamide gels together with ribonucleoproteins isolated and purified on sucrose (SG) and cesium sulfate gradients (CG). Silver staining of the gel revealed a distinct band comigrating with molecular mass markers of apparently 28 kDa.
(B) Blotting and immunostaining using antiserum against TSWV nucleocapsids identified the N nucleocapsid protein in a crude protein extract of infected tissue (TSWV) and in the fractions obtained after sucrose (SG) and cesium sulfate gradient purifications (CG). Customary or pre-stained molecular mass markers, respectively, are present in lane 'M'.

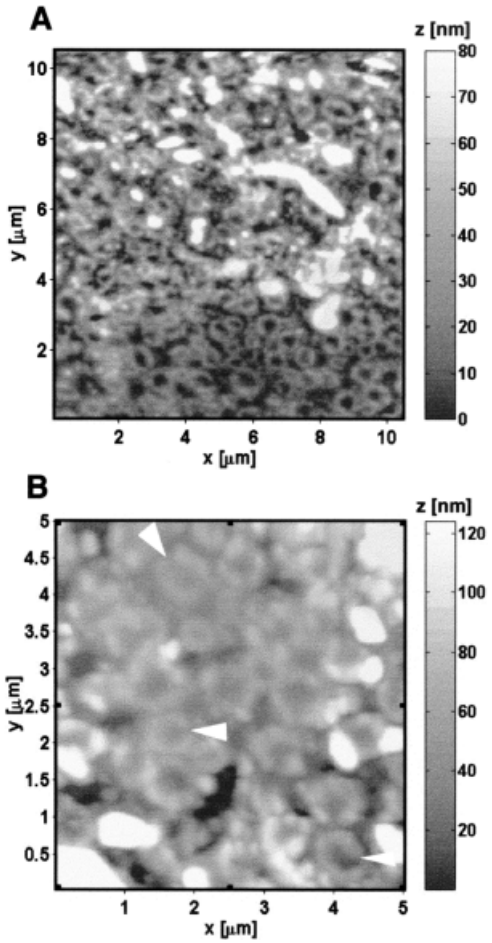

Fig. 2 AFM Images of TSWV Ribonucleoprotein Preparations.
(A) Overview of a ribonucleoprotein preparation, obtained with atomic force microscopy by scanning an array of 100 μm^2.
(B) Scanning of a 25 μm^2 area of the same aliquot. Constrictions of exemplary rings are indicated by white arrowheads.

In order to perform AFM, an aliquot of the cesium sulfate purified nucleocapsid preparation (1 µg of protein) was applied to a glass surface and air dried. The nominal tip radius of the atomic force microscope was < 50 nm, and the force constant was 0.032 N m^{-1}. After scanning of an array of about 100 μm^2, rings or ellipses of different sizes could be visualized, and scanning of an 25 μm^2 array revealed that many molecules showed one predominant constriction (Figure 2). Determination of the size of individual nucleocapsid particles revealed that three clusters of ribonucleoproteins were present in the preparation, showing a length of 1300, 1750 and 2200 nm on average of the circularized filaments (Table 1). In an attempt to resolve the third dimension of the nucleocapsids, *i. e*. the thickness or cross-section dimensions of the ribonucleoprotein filaments, the difference of the color code, which corresponds to the height of the scanned structures, was analyzed. Using line measurement software, heights between 7 and 25 nm were determined (Figure 3). However, in contrast to measurements of filament lengths, the determination of the third dimension of the structures turned out to be more difficult, possibly due to the sample preparation, surroundings of the respective nucleocapsid, recording conditions and precision of the line measurement method.

Discussion

The application of AFM to investigate the topography of *Tospovirus* ribonucleoproteins confirmed their filamentous structure. Interestingly, our AFM studies revealed regularly shaped rings, many of them constricted at one position. To our knowledge, such defined forms of nucleocapsids have not been identified using transmission or scanning electron microscopes. These methods often reveal slightly condensed or amorphous structures of ribonucleoprotein aggregates (Mohamed, 1981; Goldbach and Peters, 1996). Intriguingly, this ring structure matches the proposed pseudocircular, panhandle structure of encapsidated genomic RNA, due to the complementary

Table 1 Determination of Ribonucleoprotein Dimensions.

Cluster	Number of particles (n)	Average length of particles (nm)	Standard deviation[a] (nm)	Length of genomic RNA segments[b] (nucleotides)
I	45	1300	160	2916 (S)
II	48	1750	100	4821 (M)
III	46	2200	210	8897 (L)

Three groups of nucleocapsids with different circumferences (lengths) could be distinguished after evaluation of 139 individual particles observed by atomic force microscopy.
[a]Root-mean-square deviation of the sampling.
[b]Accession numbers: D00645, S48091 and D10066 (Goldbach and Peters, 1996).

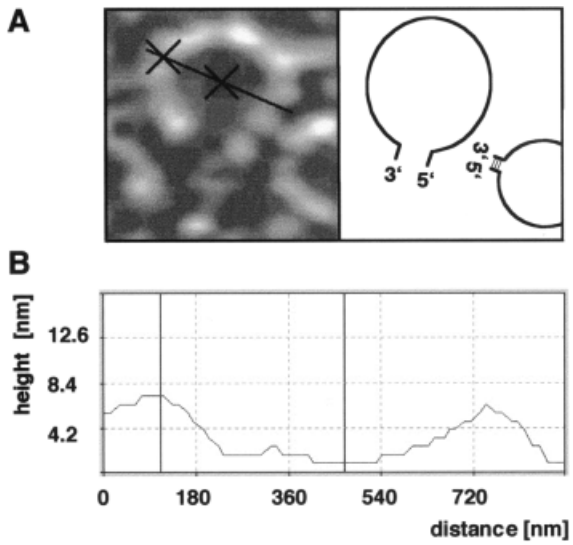

Fig. 3 Determination of the Dimensions of a Selected TSWV Ribonucleoprotein Complex.
(A) Computer line measurements, as shown in this example by the black line crossing the central area of a selected ribonucleoprotein ring, were the basis for measurements of their dimensions. The right panel depicts a schematical drawing of proposed RNA panhandle structures and how they could be located in the ribonucleoproteins shown on the AFM micrograph to the left.
(B) Example of a plot resulting from a measurement as described in (A) to determine the cross-section diameter of a selected ribonucleoprotein.

5' and 3' ends of the RNA segments (right panel of Figure 3A). Although it cannot be ruled out that the double-stranded RNA panhandle itself could be too small to be resolved with AFM, it is not unlikely that the panhandle of the circularized RNA is located at these constrictions, because it was found recently that the N nucleocapsid protein of TSWV binds cooperatively to single-stranded RNA but not to double-stranded RNA *in vitro* (Richmond *et al.*, 1998), which could lead to the gap within the observed nucleocapsid rings.

A comparison of our results obtained by AFM with the properties of TSWV nucleocapsids summarized in the Universal Virus Database (ICTVdB; http://life.anu.edu.au/viruses/ICTVdB/11050001.htm) revealed some differences, which could be due to altered fixation procedures used for the preparation of samples examined with electron microscopes: (i) based on electron micrographs, a broader range of filamentous ribonucleoprotein lengths (200 – 3000 nm) has been reported, and (ii) a thickness (*i. e*. the diameter of a nucleocapsid cross-section) of 2.0 – 2.5 nm was proposed, whereas in our measurements a minimum of 7 nm has been determined by estimating the height of the AFM observed ribonucleoprotein stuctures (Figure 3B). The height of the nucleocapsids as determined with AFM should actually not be overestimated, because it has been shown that by performing AFM on proteins in air the heights of given structures could be rather underestimated (Kasas *et al.,* 1997; Kiselyova *et al.,* 2001). Therefore, we suppose that the cross-section diameter of 7 nm, as exemplified in Figure 3, represents a minimum range of nucleocapsid thickness. Hence, calculations of the cumulative volume of ribonucleoproteins, assuming that they are in a cylindric shape, and calculations of the volume of enveloped TSWV virions, assuming that they are spherical, seem to be reasonable. The sum of ribonucleoprotein lengths as measured with AFM (Table 1) is 5250 nm, the cross-section diameter of the molecules was found to be at least 7 nm. This results in a total volume of 2.0×10^{-22} m^3. Enveloped virions containing this volume would have a diameter of 72 nm. Because mature TSWV particles were found to have a diameter of 90 nm on average [Goldbach and Peters, 1996; Database of the International Committee for Taxonomy of Viruses (ICTV): http://life.anu.edu.au/viruses/ICTVdB/11050001.htm], it is not impossible that nucleocapsids with cross-section diameters larger than 2.5 nm, as determined by electron microscopy, could be also enveloped in mature TSWV virus particles. However, it should be noted that cross-section diameters of 25 nm at a maximum, which also have been determined with AFM, would need a sphere volume of 2.6×10^{-21} m^3, resulting in a diameter of 170 nm of mature virus particles. Because particles of this size have not been observed so far, this indicates that our measurements of nucleocapsid thickness are still too imprecise, and that detailed investigations with *e. g.* ribonucleoproteins spreaded on mica or attempts to determine the effects of dilution of the preparation and the usage of different buffers are needed to resolve these disagreements.

Table 1 shows that three clusters of ribonucleoproteins could be observed with AFM which differ in their respective lengths. If an interaction between the N nucleocapsid protein and the genomic RNA would induce a fully extended state of the RNA segment, it should be expected that the ratios between the three determined ribonucleoprotein lengths match the different numbers of nucleotides of the three RNA segments (as listed in the last column of Table 1). Because our measurements revealed that the lengths of the three clusters of ribonucleoproteins observed with AFM were not equivalent to the respective lengths of linear RNA molecules (length ratios: 1.0:1.3:1.7 *versus* nucleotide number ratios 1.0:1.7:3.0), it can be suggested that the genomic RNA could appear in a compacted, presumably coiled or supercoiled form when encapsidated with the N protein. Interestingly, examinations of nucleocapsids belonging to the *Bunyavirus* genus revealed similar results, *i. e*. the ratios between the three RNA segments were 1.0:4.8:7.5 on the basis of their nucleotide numbers, whereas the respective nucleocapsid lengths showed ratios of 1.0:2.6:3.5 (Bishop, 1996, and references therein). Presumably the homotypic interaction and multimerization characteristics of the N protein of TSWV (Uhrig *et al.,* 1999) and/or different secondary structures of the RNA molecules could be the reasons for a dense packaging of RNA within the nucleocapsids.

Materials and Methods

Preparation of TSWV Ribonucleoproteins

Isolation and purification of TSWV nucleocapsids was performed according to Heinze *et al.* (1995). Fourty grams of TSWV (isolate L3, DSMZ no. PV 0182) systemically infected leaves from *Nicotiana rustica* were harvested two weeks *post* mechanical inoculation and Blendor-homogenized on ice in 3 ml × g FW^{-1} extraction buffer [10 mM Tris/HCl, pH 8, 10 mM Na_2SO_3, 10 mM EDTA, 1% (w/v) cysteine]. After filtration of crude particles through miracloth and sedimentation, the extract was centrifuged for 30 min at 10 °C and 31 000 *g*. The pellets were resuspended in ice-cold resuspension buffer [1 ml × g FW^{-1}; 10 mM Tris/HCl, pH 7.9, 10 mM Na_2SO_3, 10 mM EDTA, 1% (w/v) cysteine; 10 mM glycine, 1% Triton-X-100] and gently stirred at 4 °C for 1 h. The suspension was centrifuged for 10 min at 4 °C and 8000 *g* for sedimentation of remaining cellular particles, and the supernatant was loaded onto a 30% (w/v) sucrose cushion (sucrose resolved in resuspension buffer). After centrifugation for 1 h at 10 °C and 75 000 *g*, the pellets were resuspended in 10 mM Na_2SO_3. Cesium sulfate was added to a concentration of 0.075 g ml^{-1}, resolved, and the suspension was overlayed on a cesium sulfate cushion (1.12 g ml^{-1} in 10 mM Na_2SO_3). After ultracentrifugation (18 h, 10 °C, 116 000 *g*), an UV-light absorbing zone could be detected which was removed and diluted in 10 mM Tris/HCl, pH 7.5. After centrifugation for 2 h at 10 °C and 66 000 *g*, the pellet was resuspended in 250 µl 10 mM Tris/HCl, pH 7.5. The colorimetric assay (Bio-Rad, Hercules, USA) revealed a protein concentration of 0.4 mg ml^{-1}.

Gel Electrophoresis and Immunoblot Assays

Aliquots from crude leaf extracts and fractions obtained after ribonucleoprotein purification were loaded on 12.5% SDS-polyacrylamide gels, separated and silver stained. To detect the TSWV N nucleocapsid protein, Western blot analysis was performed. Proteins were separated on SDS-polyacrylamide gels and electrotransferred onto PVDF membranes (Roth, Karlsruhe, Germany). Polyclonal TSWV antiserum (PAB TSWV N 293), anti-rabbit IgGs conjugated with alkaline phosphatase and subsequent colorimetric reactions with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate-*p*-toluidine salts allowed the detection of respective antigens.

Atomic Force Microscopy

Aliquots of cesium sulfate gradient purified ribonucleoproteins (2.5 µl, equivalent to one µg of N nucleocapsid protein) were spotted onto a glass surface and air dried. Topographic images using atomic force microscopy were examined in air using a TMX 2000 Topometrix Explorer System (Atos, Pfungstadt, Germany) in contact mode with a tip radius < 50 nm and a force constant of 0.032 N m^{-1}. Measurements of nucleocapsid proportions as presented in Figure 3 were performed using the line measurement software package supplied by Topometrix.

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