Engineered Tobacco mosaic virus mutants with distinct physical characteristics in planta and enhanced metallization properties

Anan Kadri, Edgar Maisb, Nadja Amsharovc, Alexander M. Bittner, Sinan Balci, Klaus Kern, Holger Jeskea, Christina Wege

Universität Stuttgart, Institute of Biology, Department of Plant Molecular Biology and Plant Virology, Pfaffenwaldring 57, D-70550 Stuttgart, Germany
Leibniz Universität Hannover, Institut für Pflanzenkrankheiten und Pflanzenschutz, Herrenhäuser Straße 2, D-30419 Hannover, Germany
Max Planck Institute for Solid State Research, Heisenbergstraße 1, D-70569 Stuttgart, Germany

Article history:
Received 22 July 2010
Received in revised form 25 January 2011
Accepted 29 January 2011
Available online 15 February 2011

The publication is dedicated to Karl-Wolfgang Mundry, founder of the Plant Virology Department at the University of Stuttgart, who deceased in 2009.

Keywords:
Tobacco mosaic virus (TMV)
Coat protein
Mutation
Nanotechnology
Metallization

1. Introduction

Tobacco mosaic virus (TMV) is the type member of the genus Tobamovirus. Its virion encapsidates a helical plus-sense single-stranded RNA (+ssRNA) genome of 6395 nucleotides in 2130 identical coat protein subunits of 17.5 kDa each, and forms a rigid helical tube with a length of 300 nm, a diameter of 18 nm, and a central channel 4 nm in width. As this structure exposes several distinct and repeated functional groups on the inner and outer surfaces (Clark and Orlova, 2010; Klug, 1999; Namba et al., 1989; Sachse et al., 2007) it is suitable for differential modifications such as metallization and has therefore been the subject of intensive studies in the field of nanotechnology as a versatile biotemplate, yielding an ever increasing number of close-to-application materials and devices (e.g. Balcı et al., 2007, 2008, 2009; Dujardin et al., 2003; Gerasopoulos et al., 2010; Górzny et al., 2010; Knez et al., 2002, 2003, 2004a,b, 2006; Kobayashi et al., 2010; Lee et al., 2006; Lim et al., 2010; Miller et al., 2007; Rong et al., 2009; Royston et al., 2008, 2009; Schlick et al., 2005; Shenton et al., 1999; Smith et al., 2006; Tseng et al., 2006; Wu et al., 2010a; Yang et al., 2010; Yi et al., 2005).

TMV exists in a great number of different strains and mutants and accumulates to high levels in many host plants with variable symptom responses (reviewed in Culver, 2002; Zaitlin and Israel, 1975). Dependent on both the virus strain and the host genome, it may either spread systemically, or may be restricted to a local infection site, and induce yellowing, mosaic, severe systemic necrosis, or local necrotic lesions (Mundry et al., 1990; Zaitlin and Israel, 1975). Wild type (wt) virions are exceptionally stable under a broad range of chemical and physical conditions (Mutombo et al., 1992; Perham and Wilson, 1978; Zaitlin, 2000) and easily isolated from plants, but changes in the coat protein sequence may disrupt particle stability and impede purification.

© 2011 Elsevier B.V. All rights reserved.
Fig. 1. Constructs and sequences used. Genetic maps at different scales for the infectious wtTMV plasmid construct p843pe35TMVr.1 (A) or the mutagenesis sub-clone pAK13 (B), and alignment of the TMV CP amino acid sequences (C). Genetic elements and restriction sites used in cloning strategies, mutagenesis and to generate TMV CP-specific probes are indicated: RP, RNA-dependent RNA polymerase; MP, movement protein; CP, coat protein; OAS, origin of assembly. The resulting transcripts for the full-length TMV genome and the TMV fragment (nts 5433-6306; NC 001367) are indicated by black arcs. Alignment of the CP amino acid sequences (C) deduced from the nucleotide sequences of the indicated constructs.

The virion structure (Clare and Orlova, 2010; Namba et al., 1989; Sachse et al., 2007) provides some explanation for its stability. Carboxyl–carboxylate interactions tighten adjacent coat protein subunits, and carboxylate–phosphate pairs bind protein subunits to RNA (reviewed in Culver, 2002). Upon infection, virions face physiological conditions with lower calcium concentrations and higher pH values than in the extracellular environment, and protons as well as calcium ions are removed from the carboxyl–carboxylate and carboxylate–phosphate pairs. The resulting repulsive negative electrostatic charges destabilize the subunits’ interactions (Caspar, 1963; Caspar and Namba, 1990), and permit disassembly necessary for translation and replication. Hence, one of the so-called “Caspar-carboxylate” groups (glutamic acid at position 50) was changed to glutamine in this study, in order to stabilize the rod structure and prevent its disassembly as described (Bendahmane et al., 2007; Culver et al., 1995; Lu et al., 1996), to improve the robustness of the biotemplate for metallization purposes.

In addition, amino acids may be replaced or inserted at the C-termini, the N-termini, or within the exposed loop in order to functionalize the outer surface of the virion specifically (Lee et al., 2005, 2006; Smith et al., 2006; Yi et al., 2005). Thus, in order to exploit the well-known binding affinity of histidine to nickel, cobalt or zinc, a hexahistidine oligopeptide was fused to the C-terminus of the coat protein. The stabilities and metal-binding properties of both mutants compared to those of wtTMV virions are discussed.

2. Materials and methods

2.1. Construction of infectious clones for TMV coat protein mutants

cDNA was generated from the RNA of wt full-length TMV vulgare strain (PV0107; DSMZ, Germany), inserted into the bacterial plasmid pT7T3 19U (Pharmacia) between a duplicated Cauliflower mosaic virus 35S promoter (Restrepo et al., 1990) and a hammerhead ribozyme (Shintaku et al., 1996) using standard cloning protocols (Sambrook and Russell, 2001), resulting in the plant-infectious plasmid p843pe35TMVr.1 (Fig. 1A).

To generate mutants, a sub-clone (pAK13, Fig. 1B) comprising nucleotides 5433 to 6306 of TMV cDNA was raised from p843pe35TMVr.1 using pBluescript-SKII+(Stratagene, Heidelberg, Germany) as vector: the corresponding 874 bp cDNA fragment was amplified by PCR [95°C, 5 min; 25 cycles 95°C, 30 s, 72°C, 30 s, 72°C, 1 min; 72°C, 10 min], using Taq DNA polymerase (Qiagen, Hilden, Germany), forward primer #1 and reverse primer #2, each adding an XhoI restriction site (Table 1), and inserted into the same restriction site of the vector following standard protocols (Sambrook and Russell, 2001). Mutants were raised by PCR amplification of the entire sub-clone pAK13 in the presence of mutant primers and blunt-end re-ligation, for pAK-6xHis using Taq DNA polymerase (Qiagen) with primers #3 and #4 [95°C, 5 min; 25 cycles 95°C, 30 s, 72°C, 30 s, 72°C, 3 min; 72°C, 7 min], or for pAKE50Q using proofreading DNA polymerase (Qiagen) and primers #5
Table 1
PCR primers used for TMV CP cloning, mutagenesis, sequencing, or RT-PCR.

<table>
<thead>
<tr>
<th>#</th>
<th>Name</th>
<th>Sequence* (5′ to 3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5'AKp843TMVxhoel5433a</td>
<td>CCAACTTCGAGGATTTAACAACGGAGCAGGAGG</td>
</tr>
<tr>
<td>2</td>
<td>3'AKp843TMVxhoel6306b</td>
<td>CCAACTTCGAGCGGCGTAAAGACACCAAGACACCCCTCG</td>
</tr>
<tr>
<td>3</td>
<td>5'AKp81CIFTMV6206Ma</td>
<td>ATATATAAAACCGAGTGGTCGTATATACTACGAGCACGGGAGG</td>
</tr>
<tr>
<td>4</td>
<td>3'AKp81CIFTMV6205Mb</td>
<td>GCATCTTGACTGCTAGATGTTGCCAGGACTAGAGG</td>
</tr>
<tr>
<td>5</td>
<td>5'AKp81CIFTMV5851Ma</td>
<td>GACACTCACTCAGGACGGAGAACC</td>
</tr>
<tr>
<td>6</td>
<td>3'AKp81CIFTMV5850Mb</td>
<td>TTAGAACGACGAGCTCGGCAGTGTTC</td>
</tr>
<tr>
<td>7</td>
<td>AKpTMVpveq5680MF</td>
<td>CCTACTGTCGCGGAACTGCGATCC</td>
</tr>
<tr>
<td>8</td>
<td>AKpTMVpveq5971.MR</td>
<td>CCAACTAACAGTCTGACGTACCC</td>
</tr>
<tr>
<td>9</td>
<td>AKpTMVpveq362.F</td>
<td>CGGTTTCTCTGACGTTGGT</td>
</tr>
<tr>
<td>10</td>
<td>AKpTMVpveq046.R</td>
<td>CTTGCCGTCGAGTATAGAAGC</td>
</tr>
</tbody>
</table>

* Added restriction sites are underlined. Inserted or altered sequences are bold.

and #6 [95 °C, 5 min; 25 cycles 94 °C, 45 s, 68 °C, 30 s, 72 °C, 4 min; 72 °C, 10 min]. Correctness of the resultant clones was confirmed by sequencing using a semi-automated system (LI-COR Biosciences GmbH, Bad Homburg, Germany) and IRD-labeled primers (Table 1) #7 or #8 after cycle-sequencing reactions [92 °C, 2 min; 30 cycles 92 °C, 15 s, 64 °C for primer #7 or 54 °C for primer #8, 15 s, 70 °C, 30 s].

2.2. Virus propagation and infectivity assays

Seeds of test plants were germinated in soil and maintained under greenhouse conditions with auxiliary lighting (24 °C/16 h day period and 18 °C/8 h night period). TMV CP mutants (6xHis or ES0Q) and wtTMV were propagated in Nicotiana tabacum L. cv. ‘Samsun’ nn or NN, ‘Xanthi’ nc, Nicotiana benthamiana Domin, Nicotiana rustica L. cv. ‘Pavonii’ and ‘Cordata’. Two upper leaves (No. 4 and 5) of the plants in the five to six leaf-stage were mechanically inoculated using Carborundum® as an abrasive with infectious plasmid DNA (1 μg per leaf diluted in 100 mM sodium potassium phosphate buffer [SPP], pH 7.4, prepared according to Sambrook and Russell, 2001). For lesion assays on ‘Samsun’ NN and ‘Xanthi’ nc, 1 or 0.5 μg of the corresponding infectious cDNAs was applied per half of same leaves; additionally, plant sap from symptomatic N. benthamiana upper leaves (prepared from 10 mg infected material in 100 μl SPP per inoculated leaf) was used on separate plants. Inoculated or buffer-treated control plants were maintained in a greenhouse under 20 °C/16 h light and 15 °C/8 h dark periods.

2.3. Virus enrichment and purification

Two methods for virus isolation were compared.

Method A. Slightly modifying the protocol of Devash et al. (1981), virus was purified from symptomless plants (N. tabacum cv. ‘Samsun’ nn, 14, 21 and 28 dpi) by differential and density-gradient centrifugation. Young symptomically infected leaves (20 g) were harvested and homogenized (blender) with three volumes per g fresh weight in 0.1 M EDTA pH 8.0 at 4 °C. The homogenate was filtered through two layers of gauze, and the filtrate centrifuged (5300 × g, 15 min, 4 °C, Sorvall, SLA-1500 rotor). The resultant supernatant was filtered through a 1 cm thick layer of Celite® (545, 0.02-0.1 mm grain size, Merck Biosciences, Bad Soden, Germany). The resulting filtrate was incubated for 90 min at 50 °C. Denatured plant proteins were removed by sedimentation (as above). The supernatant was centrifuged (117,000 × g, 90 min, 4 °C, Beckman, Ti60 rotor) and the sediment resuspended in 10 mM SPP (pH 7.0) using 0.5 ml per g initial plant tissue. Virus particles were separated from aggregates by low-speed centrifugation (4800 × g, 10 min, 4 °C, Sorvall, SLA-1500 rotor). The supernatant was either loaded onto sucrose gradients for rate-zonal centrifugation or for isopycnic centrifugation.

Method B. Virus particles were isolated from N. benthamiana following the procedure of Chapman (1998). Frozen (−70 °C) systemically infected leaves (5 g) from symptomless plants (21 and 28 dpi) were pulverized with mortar and pestle in 50 ml 100 mM SPP and filtered through three layers of gauze. Aliquots of each purification step were kept for further analysis and the remainder processed as follows: filtrates (F; 12.5 ml) were centrifuged (5000 × g, 15 min, 18 °C, Sorvall, SS34 rotor), from which pellets (P1) and supernatants (S1) were separated, n-butanol (final concentration [f.c.] of 8%) was added dropwise to the supernatants while stirring the mixture at room temperature (RT) for 45 min prior to its clarification by centrifugation (10,000 × g, 30 min, 4 °C, Sorvall, SS34 rotor). The resulting pellets were resuspended (P2), the upper butanol phases discarded, and the supernatants filtered through Miracloth® (S2).

2.3.1. Rate-zonal centrifugation in sucrose density gradients

Samples (0.5–1 ml) were layered onto 10–30% sucrose gradients, centrifuged (32,000 rpm, 2.5 h, 4 °C, Beckman, SW40 rotor) and fractionated into 33 aliquots into microtiter plates. Absorbance profiles (A260, A280 and A320) were recorded for the fractions and the resuspended pellets. Selected fractions were pooled (50 μl each), dialyzed against 10 mM SPP buffer at 4 °C, and analyzed by Western blotting and electron microscopy.

2.3.2. Isopycnic density-gradient centrifugation in the presence of Cs2SO4

Samples were diluted to a final volume of 13.5 ml 100 mM SPP and adjusted to 35% Cs2SO4 (w/v) and centrifuged (16,000 × g, 10 min, 4 °C, Sorvall, HB6 rotor) to remove aggregated material. The resulting supernatants were centrifuged to equilibrium density (50,000 rpm, 17 h, 20 °C, Beckman, VTi65.1 rotor) and 36 fractions of 400 μl were collected from the bottom of the tubes. Their absorbance profiles (A260, A280 and A320) were recorded using a SPECTRAFluor plus spectrophotometer (TECAN, Crailsheim, Germany) and their virus content by enzyme-linked immunosorbent assay (ELISA).

2.4. Nucleic acid isolation, RT-PCR and sequencing

Total nucleic acids were purified from young, systemically infected N. tabacum cv. ‘Samsun’ nn leaves or inoculated tobacco ‘Samsun’ NN leaves exhibiting local lesions (100 mg each), at 14, 21 and 28 dpi according to Boom et al. (1990). TMV RNA from purified TMV particles was obtained by means of phenol/chloroform extraction and alcohol precipitation.
RNA samples were reverse-transcribed using an AMV first-strand cDNA synthesis kit (Invitrogen, Karlsruhe, Germany) and TMV primer #2 (Table 1) following the manufacturer’s instructions. One tenth of the volume of cDNA (2 μl) and the primers #1 and #2 were used for PCR [95 °C, 5 min; 35 cycles of 95 °C, 45 s, 56 °C, 45 s, 72 °C, 1 min; 72 °C, 10 min]. Fragments from 15 μl samples of the PCR reaction were separated in 0.8% agarose gels in TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA), eluted and sequenced.

2.5. TMV-specific probe

To obtain a TMV-plus-strand-specific probe, pAK13 was digested with Hind III and transcribed in vitro using T7 polymerase (DIG–RNA labeling kit (SP6/T7), Roche Applied Science, Penzberg, Germany).

2.6. RNA extraction and Northern blot analysis

For total nucleic acids isolated from plant tissues, samples (15 μl) were separated on 1% agarose/MOPS gels and transferred to nylon membranes (Hybond-NX, Amersham Biosciences, NJ, USA) according to Sambrook and Russell (2001). RNA was hybridized with a DIG-labeled probe specific for TMV CP RNA, and signals were detected as above.

2.7. Polycrylamide gel electrophoresis (PAGE) and Western blot analysis

Electrophoresis and Western blotting were performed according to standard methods (Laemmli, 1970; Towbin et al., 1979). Samples were denatured by boiling in Laemmli sample buffer (100 mM Tris–HCl pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerin, 200 mM DTT), electrophoresed using 15% polyacrylamide gels, stained with Serva-Violet 17 (SERVA Electrophoresis GmbH, Heidelberg, Germany) or electro-blotted to nitrocellulose membranes (Protran BAB5, Schleicher & Schuell, Dassel, Germany). Blots were blocked with 5% (w/v) nonfat milk powder for at least 1 h at RT, incubated with TMV-specific rabbit IgG (kindly provided by Prof. K.W. Mundy, Stuttgart, Germany, diluted 1/1000) or anti-pentaHis mouse antibodies (Biotrend, Cologne, Germany). Goat anti-rabbit IgG or rabbit anti-mouse IgG conjugated with alkaline phosphatase (Biotrend, Cologne, Germany) were used as secondary antibodies. Enzyme activity was detected using NBT/BCIP (nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate, toluidine salt) or CDP-star® (Roche Applied Science).

2.8. Enzyme-linked immunosorbent assay (ELISA)

Aliquots (50 μl) of each fraction from the density gradients were analyzed by ELISA according to standard procedures using Greiner high-binding plates (Greiner Bio-One International AG, Frickenhausen, Germany) and rabbit anti-TMV IgG (1/1000 dilution) and alkaline phosphatase-conjugate (goat anti-rabbit IgG, Biotrend; 1/5000). Between treatments, plates were washed with phosphate-buffered saline supplemented with 0.05% Tween-20. After adding substrate (100 μl p-nitrophenyl phosphate, 1 mg/ml) to each well, enzyme activity was measured at A405 using a SPECTRAFluor Plus spectrophotometer (TECAN).

2.9. Electron microscopy

Samples were adsorbed on Formvar®/carbon-coated 400-mesh grids, rinsed with 40 drops of ddH2O and negatively stained with 1% aqueous uranyl acetate containing 250 μg/ml bacitracin. Immunosorbent electron microscopy (ISEM) (Milne and Lesemann, 1984) was performed by coating grids with antibodies (rabbit anti-TMV IgG or mouse anti-pentaHis monoclonal IgG (Qiagen, Hilden)) diluted 1/1000 in 10 mM phosphate buffer, pH 7 for 15 min. The coated grids were incubated with samples for 15 min at RT, rinsed with water and negatively stained as described above.

2.10. Metallization of TMV particles with nickel

Electroless metal deposition was applied to the different virus variants following a slightly modified method on the basis of Balci et al. (2006). For activation with Pd(II), samples (50 μl) from filtrates (F, see above) of wt or mutant TMV-infected plant material, respectively, were diluted with H2O (250 μl), dialyzed twice against H2O (10 min, Slide-A-Lyzer 10,000 MWCO, incubated (10 min, RT) with an equal volume of freshly prepared activation bath (1.36 mM Na2PdCl4, 1 M NaCl) at pH 7.0 and centrifuged (13,000 rpm, 5 min, Eppendorf, 5417C rotor). The resulting brownish pellets were washed with H2O, sedimented (13,000 rpm, 5 min, Eppendorf, 5417C rotor) and suspended in 300 μl H2O. For nickel electroless deposition, the Pd-activated suspensions were mixed with an equal volume of metallization solution (223 mM Ni(CH3COO)2·4H2O, 228 mM lactic acid, 42.4 mM (CH3)2NH·BH3, adjusted to pH 7.5 with NaOH). Aliquots were taken as soon as metal was observed, immediately adsorbed on Formvar®/carbon-coated 400-mesh grids, incubated for 2 min and analyzed by electron microscopy.

2.11. MALDI-MS analysis

Systemically infected leaves (200 mg) from symptomatic plants (21 dpi) were pulverized with mortar and pestle in 2 ml 100 mM SPP and filtered through three layers of gauze; samples from each filtrate (F), pellet (P1 and P2) and second supernatant (S2) were prepared for MALDI-MS analysis. Viruses were denatured by adding 6 μl guanidine hydrochloride (6 M) to the sample (24 μl) and mixing for 5 min at RT. Denatured proteins were spotted on MTP 384 massive target plate using Millipore ZipTips®C18® tips (Millipore Corporation Billerica, USA) to remove excess salts and assist the binding of protein to the sinapic acid matrix (Sigma–Aldrich, Germany). MALDI-TOF-MS analysis was performed using a Bruker reflex IV mass spectrometer (Bruker Daltonics, Germany) and linear positive mode.

3. Results

3.1. Infectious constructs

The TMV CP gene was amplified from the infectious full-length construct p843pe35TMVr.1 (Fig. 1A), and TMV CP mutants 6xHis (hexahistidine at the C-terminus) and E50Q (amino acid substitution at position 50) were generated in pAK13 (Fig. 1B), giving pAK-6xHis and pAK-E50Q. Their inserts were transferred back in place of the wt CP gene of the infectious construct, giving pTMVcp6xHis and pTMVcpE50Q. The first resulting clones carried an unintended second mutation (S123N) in pAKcp6xHis-1, which
Numbers and diameters of local lesions on N gene-carrying tobacco induced by infectious plasmid DNA, or by plant sap from systemically infected N. benthamiana leaves for different TMV variants, analyzed at 7 or 2 days post inoculation, respectively.

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Infectious CDNA</th>
<th>Plant sap</th>
</tr>
</thead>
<tbody>
<tr>
<td>Host</td>
<td>Samsun NN</td>
<td>Xanthi nc</td>
</tr>
<tr>
<td>TMV variant</td>
<td>LL</td>
<td>D</td>
</tr>
<tr>
<td>wtTMV</td>
<td>2.7</td>
<td>0.5–2.0</td>
</tr>
<tr>
<td>TMV-ESQ</td>
<td>2.9</td>
<td>0.5–2.0</td>
</tr>
<tr>
<td>TMV-His</td>
<td>1.2</td>
<td>0.2–1.0</td>
</tr>
<tr>
<td>TMV-His+ (*S123N)</td>
<td>0</td>
<td>–</td>
</tr>
</tbody>
</table>

Average numbers of local lesions, and ranges of their diameters on locally infected leaves of the indicated hosts. LL: local lesions per leaf (decimals omitted for values >10); D: diameter in mm.

was absent from a second construct (pAKcp6xHis-2) generated by use of a proofreading DNA-polymerase (Fig. 1C, and sequencing data not shown). Both variants of the 6xHis mutant behaved similarly in the subsequent analysis, except for a distinct response induced in local lesion hosts (see below), indicating that the S123N exchange did not have any detectable influence on properties of the mutants’ systemic infection and physicochemical characteristics of the virions.

All constructs were inoculated onto plants for virus propagation. Initial infectivity assays were carried out with Nicotiana tabacum ‘Samsun’ nn or NN (referred to as nn, NN tobacco in the following). Following mechanical inoculation of plasmid DNA, E50Q induced typical systemic mosaic symptoms on nn tobacco like wtTMV (not shown), but delayed (at 21 dpi compared to 14 dpi for wtTMV). However, mosaic development stopped later on, and no symptoms were observed on newly developed leaves beyond 40 dpi. On the inoculated leaves of nn and nc tobacco, E50Q-infected local induced lesions, the number and size of which did not significantly differ from those elicited by wtTMV plasmid (see Table 2). 6xHis revealed inconspicuous up to mild symptoms at 21 dpi on the systemic host. A lower number of lesions of slightly different diameter on plants with the N gene were generated by 6xHis (Table 2). The clone pAKcp6xHis-1 harboring the second-site CP mutation S123N, however, did not yield any lesions. A second set of local lesion assays was carried out using plant sap from systemically invaded N. benthamiana leaves as inoculum. These results (Table 2) confirmed that the accurate 6xHis mutant provoked less local lesions than wtTMV (in contrast to the 6xHis + S123N clone lacking lesions). Plant sap from E50Q-infected N. benthamiana produced only negligible numbers of local lesions in the same assay.

Total RNAs were isolated from leaves with local lesions from NN, and from systemic leaves from nn tobacco and analyzed by RT-PCR and sequencing. For E50Q, viral RNA was successfully obtained from lesions on NN plants, but was below the detection limit in systemic tissues of nn plants, which, in contrast, provided RNA readily when the TMVcp6xHis-2 plasmid was used as inoculum. Direct sequence analysis of the RT-PCR products confirmed that the 6xHis as well as the E50Q progeny had retained the engineered mutations and had not undergone revertant or second site CP mutations (data not shown).

wtTMV particles were successfully isolated from N. tabacum nn by use of method A, whereas 6xHis purification repeatedly failed, and E50Q revealed two bands after CsCl density-gradient centrifugation. The lower and fainter band complied with a buoyant density of 1.32, as for wtTMV (data not shown). The upper major band corresponded to a reduced density expected for RNA-free TMV protein assemblies. Electron microscopic analysis verified that both bands contained TMV-like particles of variable length (data not shown). In agreement with its lower buoyant density, no genomic TMV RNA was extractable from the predominant E50Q band, yielding further evidence that E50Q protein had assembled mainly into RNA-free particles.

### 3.2. Virus propagation and symptoms in different host plants

Since the initial purification of 6xHis from N. tabacum nn was insufficient, additional hosts were tested. Following mechanical inoculation with plasmid DNA, both mutants induced systemic symptoms (vein clearing and leaf curling) in N. benthamiana from 7 dpi on, which frequently developed to severe symptoms (14 dpi) and systemic necrosis at 28 dpi (Fig. 2). Symptoms varied and were even more enhanced in several combinations of the virus variants with different N. rustica types (Fig. 2). In the wild N. rustica variant, 6xHis or wtTMV provoked stunting and leaf necrosis, whereas E50Q primarily affected stems, resulting in death of adjoining leaves (Fig. 2). In the cultivar ‘Cordata’, pronounced stunting was observed with wtTMV and 6xHis, the latter of which also caused severe necrosis. By contrast, E50Q induced only few yellowing spots in systemically invaded leaves (Fig. 2). Finally, N. rustica ‘Pavonii’ showed similar symptoms for all virus variants, including stunting and expanded necrotic areas of systemic leaves (Fig. 2).

### 3.3. Virus isolation

N. benthamiana was used as an alternative host for isolation of the 6xHis mutant. Method A was applied for differential centrifugation, and combined with rate-zonal sucrose-gradient centrifugation. wtTMV was treated in parallel. As analyzed by Western blotting, part of the viral 6xHis particles accumulated in the same gradient fractions as wtTMV, but different from it, as the majority pelletted through the gradient, indicating a high tendency to aggregate for the mutant virus (data not shown). Upon SDS polyacrylamide gel electrophoresis, the 6xHis mutant exhibited considerable amounts of CP dimers and trimers retained in the gels, with apparent molecular weights of 36 and 54 kDa, respectively (data not shown).

Due to the differences in the behavior of the 6xHis particles compared to wtTMV during purification, method B was chosen as an alternative enrichment technique for all TMV variants. All intermediate fractions were scrutinized for the presence of viral particles by TEM, and the yields of the coat proteins from various plants and purification steps were quantified in two independent experiments using comparative and standardized Serva-violet 17 stained gels. Filtrates (F), pellets (P1), pellets (P2) and supernatants (S2) for wtTMV-, 6xHis- or E50Q-infected N. benthamiana plants were collected. In the filtrates, abundant CP was detected on Western blots for all TMV types (Fig. 3). wtTMV particles accumulated in the supernatant fractions S2 after butanol clarification (Fig. 3A), which was in contrast to both mutants that were mainly present in pellets (Fig. 3A; P1, P2). The majority of 6xHis protein (67/71% in two independent experiments, Table 3) was found in P1, whereas the majority of E50Q protein (54/63%, Table 3) was present in P2. Detection of the 6xHis CP extension with anti-His tag antisera confirmed the predominant presence of this protein in pellets P1 (Fig. 3B), and the maintenance of the CP modification.
Fig. 2. Symptoms in different Nicotiana species or cultivars as indicated, after infection with the construct variants at 28 dpi. Note the high necrotic potential of 6xHis in different hosts, which may interfere with efficient virus purification. For details, refer to text.

Mass spectrometry (MALDI-MS) was used to analyze whether the His-tagged protein population was homogeneous. The analysis confirmed the unique presence of only one type of coat protein in each virion preparation, as obvious from single distinct mass peaks for every preparation (Fig. 3C). In order to further scrutinize the sequence identity of the viral progenies, plants were inoculated with the different variants (27 plants in total per virus in three independent experiments), total RNA was extracted from systemic leaves at 21 dpi, RT-PCR was applied to bacterially clone the CP cDNA, and 20–25 colonies were randomly selected for each variant and their insert DNA sequenced. The sequence analysis confirmed the sole presence of the intended mutants and ruled out any kind of reversion (data not shown).

3.5. Variant particles after isopycnic density gradient centrifugation

Filtrate, pellet and supernatant fractions obtained from method B were processed in parallel for isopycnic Cs2SO4 centrifugation and the resulting fractions were analyzed by ELISA using anti-TMV antibodies (Fig. 4). The corresponding densities of the fractions were determined by refractometry, and ELISA values were drawn to the density scale. wtTMV particles with 5% RNA accumulated at densities of 1.29–1.33 g/cm³ (Fig. 4; wtTMV; major peak), whereas RNA-free TMV protein was found in the upper fractions with densities of 1.25–1.27 g/cm³ (Fig. 4; wtTMV; minor peaks). Most of wtTMV was retrieved from S2 as expected. Of the 6xHis particles from the first pellet fraction (P1), a small portion migrating at a density of 1.3 g/cm³ indicated that at least some particles were formed with the canonical concentration of 5% RNA. However, the majority of their proteins were found in a broad distribution over the whole of the gradients, indicating that these particles are heterogeneous and have a tendency to decompose and/or aggregate with host components. E50Q protein, as expected from the previous experiments, was mainly found in RNA-free fractions with (Fig. 3E), E50Q-RNA was detectable as faint bands in filtrate (F) and S2.

### Table 3

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Purification steps according to method Ba</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F 1 2</td>
</tr>
<tr>
<td>wtTMV</td>
<td>100 100</td>
</tr>
<tr>
<td>TMV-His6</td>
<td>100 100</td>
</tr>
<tr>
<td>TMV-E50Q</td>
<td>100 100</td>
</tr>
</tbody>
</table>

a Values denote percentage amounts of TMV CP in systemically infected *N. benthamiana* leaves at 21 dpi in two independent experiments. TMV CP in the indicated fractions was quantified by means of dilution series analyzed via comparative SDS–PAGE after Serva-Violet staining. Absolute yields varied between experiments from 1.2 to 18 mg per g plant fresh weight with no significant differences between TMV variants.
Fig. 3. Detection of CP and RNA for the four TMV variants during enrichment procedure (method B). Western blot (A, B), mass spectrometry analysis (C) and Northern blots (D and E) are shown for two independent N. benthamiana plant samples (lanes #1 and #2). Plants were inoculated with 6xHis (H), E50Q (Q) or wtTMV (T). Mock-inoculated N. benthamiana plants (C) served as negative control. Filtrates (F), supernatants (S2) and pellet fractions (P1) and (P2) are compared. Proteins were detected either with anti-TMV antibodies (A and B) or anti-pentaHis antibodies (B and D). M: prestained molecular weight marker (NEB). The calculated sizes of TMV CP (A) or His-tagged TMV CP (B) are indicated. MALDI-TOF spectra (C) of the respective proteins are indicated by the abbreviation of the variant and purification step from which they stem. Molecular masses are indicated above the single peak signals. RNA samples (D and E) were separated on SDS-agarose gels, blotted onto nylon membranes, and viral RNA was detected with a plus-strand-specific TMV CP RNA probe. (D) and (E) display the same blots with different exposure. The length of the TMV genome (6.4 kb) is indicated.
Fig. 4. Distribution of virions after different enrichment steps followed by isopycnic gradient centrifugation. *N. benthamiana* plant samples infected with the indicated TMV variants were processed by method B and filtrates (F), pellets (P1 and P2), and supernatants (S2) were collected. The respective fractions were separated in C5SO4 density gradients. Viral proteins were detected using anti-TMV antibodies in ELISA assays (absorption at 405 nm). Cesium densities of every second fraction were determined by refractive index measuring and interpolated for all fractions and used as ×scale [g/cm³]. The densities of prominent peak fractions are indicated [g/cm³].

3.6. Electron microscopy

The fractions obtained by method B were analyzed by negative staining and electron microscopy (Fig. 5). Rodlike 6xHis particles were present in the filtrate (data not shown) and in P1 (Fig. 5A), and tended to cluster considerably. E50Q particles were found in the filtrate (Fig. 5B) with extended length, frequently exceeding 2 μm. However, after butanol treatment, clumps of E50Q CPs accumulated and interfered with the detection of a low number of virus-like, but mostly short particles (P2 in Fig. 5C). wtTMV, as expected, revealed large amounts of typical helical rod-shaped particles about 300 nm in length in S2 (Fig. 5D). For E50Q, a few helical particles were found in P1 (not shown) and in S2 (Fig. 5E). Rare 6xHis virions with the shape of TMV were observed in P2 and S2 (Fig. 5F).

3.7. Differential metallization of variants

Filtrate-derived virus particles from wtTMV and both mutants (isolated via method B) were subjected to metallization reactions. After dialysis against water and subsequent electroless reductive nickel deposition (Knez et al., 2002), samples were analyzed by electron microscopy. Under the reaction conditions applied, nanowires up to 100 nm in length and with a diameter of 3 nm were formed within the central channel of the majority of E50Q particles, exceeding the number as well as the length of the wires produced in wtTMV in parallel (Fig. 6A–C). The length distributions of the nickel wires (Fig. 6E) showed a clear superiority of E50Q mutant-over wt TMV-templating.

The surface of the 6xHis particles directed a dense deposition of large nickel clusters to the outside of the virions (Fig. 6D). All particles resolved were covered completely with these metal clusters. A metallization of the outer surface has never been achieved with wtTMV in the absence of phosphate (Knez et al., 2004b) so far, hence the result is another clear indication for substantial changes in the viral 6xHis particles’ surface chemistry.

4. Discussion

Distinct wild type or genetically modified full-length TMV cDNA constructs were suitable to infect various host plants by mechanical inoculation. In contrast to a previous report (Dagless et al., 1997), infection was efficiently achieved via mechanical inoculation of diverse *Nicotiana* species with viral cDNA, the transcription of which was driven by a 2x35S *Cauliflower mosaic virus* (CaMV) promoter. A maximum of 1 μg DNA per plant was sufficient to induce infection. As described for TMV in general (Zaitlin, 2000; Zaitlin and Israel, 1975) and for its mutants in particular (Culver, 2002; Mundry et al., 1990), the same inocula resulted in diverse plant responses, depending on the type and physiological state of the hosts. TMV CP amino acid alterations are known to contribute specifically to symptom determination, namely to the induction of chlorosis and necrosis (reviewed in Culver, 2002; Dawson, 1999). Necrotic Hypersensitive-Like Responses (HLR) occurring in otherwise systemic TMV hosts are a frequent obstacle upon the production of peptide-expressing or –coated TMV vectors, and recent evidence suggests mechanistic relations to HR such as mediated by the
N gene (Li et al., 2010b), but specific molecular signaling events have not been identified so far. It is often triggered by insertions at the carboxy terminus of the CP increasing its isoelectric point (Bendahmane et al., 1999). Consistent with these findings, both TMV variants 6xHis and E50Q elicited enhanced necrosis in some of the hosts (Fig. 2). For the production of TMV-based nanotemplates adapted to diverse current and forthcoming applications in biomedicine (Frolova et al., 2010; Li et al., 2010a; Smith et al., 2006), materials science (Soto and Ratna, 2010; Young et al., 2008), and technical sensing or catalytic devices (Gerasopoulos et al., 2010; Mao et al., 2009), it is therefore necessary to choose the hosts with the mildest symptoms, and to harvest the products early after infection. In the current study, N. benthamiana performed best amongst a number of plant types tested.

A C-terminal hexahistidine tail did not interfere with the structure of virions assembled in planta to a detectable extent, as has been reported for other short C-terminal insertions into TMV CP (Bendahmane et al., 1999). The E50Q mutation altering the Caspar-carboxylate composition stabilized the particles, whereby the capacity of systemic spread was reduced, as demonstrated previously (Culver et al., 1995; Lu et al., 1996); hence symptom development was delayed, and homogenates of systemic leaves did not induce HR in local lesion tests. E50Q proteins assembled mainly without the contribution of RNA but with extended length, consistent with former findings (Culver et al., 1995; Lu et al., 1996). Accordingly, the vast majority of the E50Q particles from systemically invaded tissues showed isopycnic densities (Fig. 4) characteristic of RNA-free assemblies (Zaitlin, 2000). How-
disassembled under the high salt conditions. This may indicate that a subfraction of the 6xHis particles exhibited the same buoyant packaged RNA. In isopycnic density gradient centrifugation (Fig. 4), self-assembly and revealed particles which resembled wtTMV and clearly, our hexahistidine fusion to the C-terminus did not prevent protein helix in a suitable physicochemical environment (Culver D77 residue and contributes to the destabilization of the nucleo-

ever, a small amount of E50Q particles that escaped degradation when treated with n-butanol, and thus was retained in the supernatant S2, was found to encapsidate RNA and to be approximately 300 nm in length. These particles presumably contained viral full-length RNA after systemic spread of the construct. The majority of E50Q particles had an increased length, which reflects the reduced repulsion between otherwise more negative electrostatic patches of adjacent CP subunits in this mutant lacking the carboxyl group of E50, which in wild type TMV is in close proximity to that of the D77 residue and contributes to the destabilization of the nucleo-

Fig. 6. TMV-based biotemplates after nickel metallization. Transmission electron microscopy in positive contrast for E50Q (A and B), wtTMV (C), and 6xHis (D). Method B filtrate fractions were adsorbed to grids after electroless deposition of nickel. Bars represent 100 nm. The length-distributions of nickel wires in the central channel for E50Q and wtTMV are compared (E). Histograms represent the percent-

In conclusion, this study demonstrates that genetic engineering of the tubular plant virus has resulted in new types of biotemplates that governed the final nanostructure of inorganic reaction prod-

In summary, despite of limitations upon the formation of homogeneous nucleoprotein nanotubes, both TMV CP mutants did not only allow accumulation of virion titers sufficient for subsequent applications in the inoculated leaves, they also were able to mediate long-distance (LD) transport of infectious nucleic acid via the vascu-

particles showed a high tendency to aggregate (Fig. 5A), which might be explained by an interaction of the His-tag with host nucleic acids and has been observed for further His-tagged protein species (Renz et al., 2006). In addition, the 6xHis mutant induced necrotic reactions (Fig. 2) with the consequence of polyphenol accumulation and crosslinking of proteins. In this view, it is conceivable that large portions of dimers and multimers of 6xHis CP remained stable during SDS–PAGE (Fig. 3A). Interestingly, the TMV 6xHis CP, if expressed in yeast, did not assemble with RNA in vitro (Mueller et al., 2010).

As with our previous studies (Balci et al., 2006; Knez et al., 2002, 2003, 2004b), the new TMV variants were successfully met-

Therefore, the 6xHis mutant was metallized specifically on its outer coat without any further treatment, where Pd(II) was bound to the His-tags. On the outer surface of wtTMV, nickel accumulation can be induced by phosphate conditioning (Knez et al., 2004b). In contrast, for wtTMV and the E50Q particles, Pd is predominantly directed into the channel promoting Ni nanowire growth (Knez et al., 2002). Interestingly, the wires inside the E50Q mutant particles were significantly longer and formed with increased efficiency in comparison to those inside wtTMV virions under the conditions applied (Fig. 6E). The channel chemistry should be identical for both virus variants, as the E50Q mutation affects inaccessible parts of the virions. However, probably due to their enhanced stability against chemical and physical changes, the in many cases very long (up to some micrometers) virus-like tubes seem to allow for a largely unhindered growth of metal wires of improved quality. Assumedly also the absence of the RNA scaffold renders beneficial conditions for reductive nickel deposition.

In conclusion, this study demonstrates that genetic engineering of the tubular plant virus has resulted in new types of biotemplates that governed the final nanostructure of inorganic reaction prod-

However, probably due to their enhanced stability against chemical and physical changes, the in many cases very long (up to some micrometers) virus-like tubes seem to allow for a largely unhindered growth of metal wires of improved quality. Assumedly also the absence of the RNA scaffold renders beneficial conditions for reductive nickel deposition.

In conclusion, this study demonstrates that genetic engineering of the tubular plant virus has resulted in new types of biotemplates that governed the final nanostructure of inorganic reaction prod-

Oligopeptides can be introduced on the outer face of TMV CPs without altering protein folding (Negrouk et al., 2004). Similarly, our hexahistidine fusion to the C-terminus did not prevent self-assembly and revealed particles which resembled wtTMV and packaged RNA. In isopycnic density gradient centrifugation (Fig. 4), a subfraction of the 6xHis particles exhibited the same buoyant density as wtTMV, but our data suggest that a significant portion disassembled under the high salt conditions. This may indicate that the hexahistidine tag reduced the virion stability. Moreover, the
stiff helical capsids may offer a unique ability to ultrathin interconnects for nanoscale circuits (Kwok and Ellenbogen, 2002), or to printable nanoelectronic arrays (Fan et al., 2009). Magnetic metal coatings of TMV-derived particles with pre-determined aspect ratio might contribute to a novel production route for high-performance nanotube ferrofluids applied for damping purposes in micromechanical devices: support for this concept comes from our recent results demonstrating the stable and tunable beneficial effects of tobamoviral scaffolding additives on commercially available nanosphere ferrofluids (Wu et al., 2010a,b).

Acknowledgements

The authors thank the gardeners for taking care of the plants, Sigrid Kober and Sylvia Pfeiffer for excellent technical assistance, and Katharina Kittelmann, Robert G. Milne and Carl Krill for critically reading the manuscript. This work was funded by the Baden-Wuerttemberg-Stiftung, “Kompetenznetz Funktionelle Nanostrukturen”, and subsidiarily supported by the DFG-SPP1165.

References


Nanotech. 6, 974–981.


