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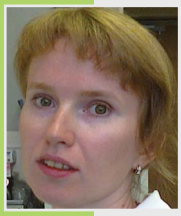

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Tatiana Komarova is a researcher working in Moscow State University, Russia. Her PhD was in molecular biology of plant viruses. Now her research is focused on the development of efficient approaches for plant-made vaccines and antibodies. Her particular interest is the design and optimization of the plant-based system for bispecific anticancer monoclonal antibody production.

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Chapter 4

Vaccine peptide display on recombinant tobacco mosaic virus particles

Tatiana V Komarova, Igor V Petrunia & Yuri L Dorokhov

Over the past several years, tobacco mosaic virus, a subject for molecular biological study and intercellular trafficking, has become a tool for the production of valuable pharmaceutical proteins. In the past 5 years, tobacco mosaic virus recombinant virions have also been developed and validated as immunogenic scaffolds, and recent studies have revealed the conditions for the efficient assembly of tobacco mosaic virus-based particles displaying HER2/neu-specific peptides on their surface.

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Aa Vaccine peptide display virus-based particles displaying vaccine epitopes on its surface

Basics about tobacco mosaic virus

Dmitrii I Ivanowsky was the first researcher to reveal that the infectious entity of the tobacco mosaic disease could pass through porcelain filters [1]. Later, Martinus W Beijerinck repeated Ivanowsky's experiments; describing tobacco mosaic disease as a *contagium vivum fluidum*, he introduced the modern usage of the word 'virus' [2]. During the 20th Century, tobacco mosaic virus (TMV) became one of the main subjects of basic study in molecular and plant biology because:

- TMV rapidly accumulates (up to 10 g/kg fresh leaf weight) and is not transmitted by insects, fungi or nematodes
- The methods of isolation are relatively inexpensive
- The virus is stable for years and even decades
- The viral particles and its coat protein (CP) are excellent immunogens

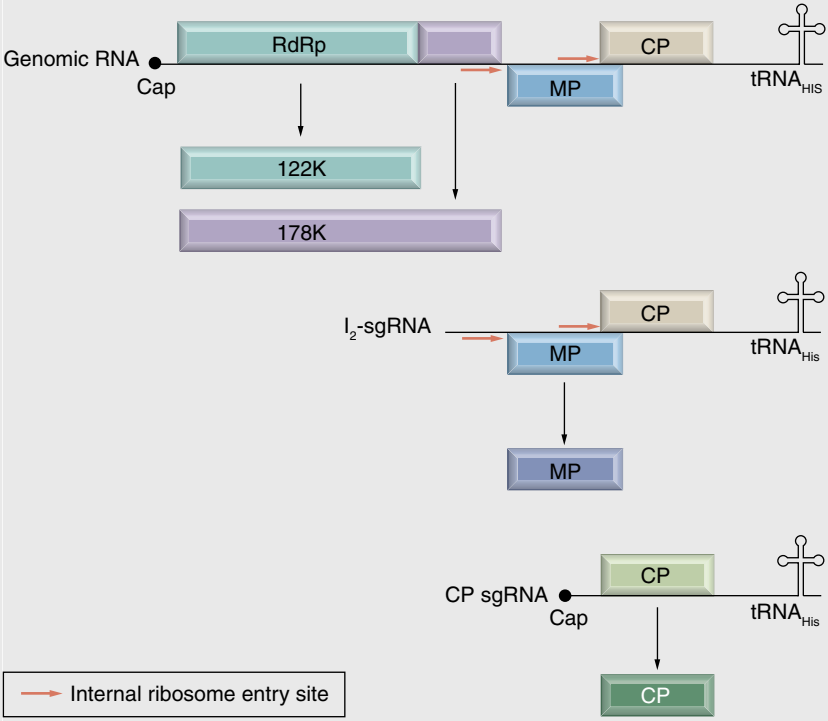
Indeed, TMV was the first virus to be used for vaccine development. Today, TMV is used as a technological tool to express valuable pharmaceutical proteins in tobacco plants.

Because the genome of the crucifer strain of the TMV has been used for the construction of the infectious cDNA vectors that are discussed in this chapter, we begin with a description of the genetic map of this TMV strain **(Figure 4.1)**. The crucifer strain of TMV genomic RNA (6312 nucleotides in length) encodes four proteins [3]. The 5´-proximal open reading frames encode a replicase subunit, a 122-kDa protein that is terminated by single amber stop codon; read-through of this termination codon results in the production of another replicase component, a 178-kDa protein. The 3´-proximal genes encode a 30-kDa cell-to-cell movement protein and a 17.5-kDa CP. The 5´-proximal genes are translated from genomic RNA, whereas the 3´-proximal genes are translated from subgenomic mRNAs derived from minus-strand subgenomic promoters, which is a key mode of gene expression for RNA viruses.

Transient expression systems

The recent success in the production of vaccines and antibodies has been based on the agroinfection technique, which provides synchronous gene expression because *Agrobacterium* is known to infect at least 96% of the cells of injected leaves simultaneously. Currently, *Nicotiana benthamiana* is the most widely used experimental host for the production of monoclonal antibodies and vaccines. An obvious advantage of transient *Agrobacterium*-based vaccine gene expression is the speed and simplicity. The full

Figure 4.1. Tobacco mosaic virus genome and strategy of expression.



The open reading frames are marked with boxes.
CP: Coat protein; MP: Movement protein; RdRp: RNA-dependent RNA polymerase; sgRNA: Subgenomic RNA.

expression of a gene of interest in agroinjected leaves may be reached within 3–4 days after being infiltrated with *Agrobacterium*.

The *Agrobacterium*-delivered TMV vectors of the first generation had a low production capacity and required the coinjection of plasmids encoding silencing suppressors, such as *Tombusvirus* p19. To adjust the TMV infectious copy transcription to the *Agrobacterium*-mediated vector delivery, a new-generation expression platform has been developed. The MagnICON system [4,5] includes the advantages of *Agrobacterium*-mediated delivery and potato virus X (PVX)-based vector and upgraded crTMV vector, in which the putative cryptic splice sites have been removed and multiple plant introns have been inserted. The system is also based on the idea of a 'deconstructed virus' (provector), by which the efficient assembly of DNA modules is obtained by recombination in

Plant viruses are not pathogenic for humans and may be used as a vaccine platform

plant cells. This system incorporates the use of *Agrobacterium*-delivered plasmids encoding the 5′-part and 3′-part of a viral vector and a plasmid encoding an integrase that is essential for full vector assembly *in planta*. For the precise assembly of the viral vector and to prevent mutations that could occur during recombination, a fragment of a plant intron has also been inserted into each part of the provector, with the donor site just upstream of the recombination site in the 5′-part and the acceptor site downstream of the recombination site in the 3′-part. Thus, the recombination site harboring possible mutations is deleted during splicing.

TMV assembly & the rational design of vaccine peptides

Tobamovirus self-assembles into a 300-nm rod-shaped particle composed of a single genomic RNA coated with an α-helical coil of 2130 CP subunits of 158 amino acids in length. Virion assembly initiates at the RNA origin of assembly, a stem–loop structure located in the movement protein gene. The current assembly model indicates that, similar to the threading of beads onto a string, TMV promotes the sequential stacking of CP discs onto the RNA until the RNA is fully encapsidated.

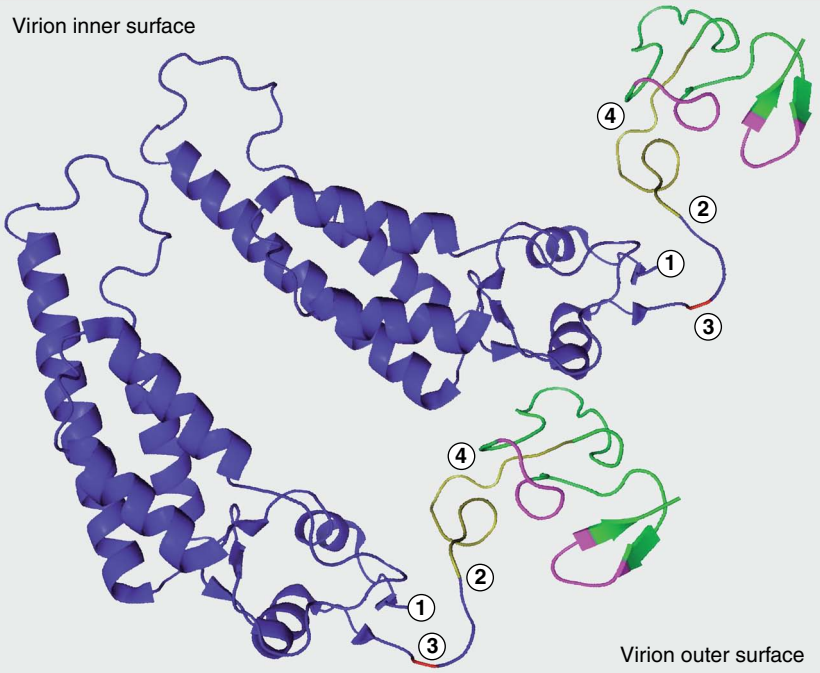
There are several advantages of presenting the antigen epitope of a candidate vaccine preparation on the TMV particles surface. First, the short peptide itself has usually low antigenic activity and is unable to initiate the high level of antibody production whereas the purified viral particles, having an adjuvant effect, properly induce the antigenic response. Second, standard virus purification protocols work well for the recombinant particles, making the vaccine preparation rapid and inexpensive. The early analysis of the secondary structure of the TMV CP revealed that the N- and C-termini were composed of surface-exposed residues that might accommodate peptide insertions. To increase the probability of recovering good yields of recombinant virions, it is important to maintain an overall neutral or negative charge of the virus particle and avoid hydrophobic peptides. Furthermore, the presence of a cysteine residue in the foreign peptide, regardless of its position and the peptide sequence, has been directly related to changes in the yield, morphology and stability of TMV particles [6,7]. In approaches using RNA transcript inoculation of tobacco, the epitopes peptides are mostly inserted near the C-terminus of the TMV CP sequence and occasionally at the N-terminus **(Figure 4.2)** **(Table 4.1)**. Direct peptide insertion into

Tobacco mosaic virus surface is an excellent vaccine epitope scaffold due to its stability, high accumulation rates and immunogenicity of viral particles

When designing a peptide display by tobacco mosaic virus, it is preferable to know physicochemical parameters of the vaccine peptide, which should be maximally approximated to those of the native coat protein

A flexible peptide linker between the coat protein and the vaccine peptide increases the efficiency of tobacco mosaic virus assembly and yield

Figure 4.2. Putative ribbon diagram of modified tobacco mosaic virus coat protein.



Two molecules of tobacco mosaic virus coat protein fused with trastuzumab binding peptide: coat protein is in purple, $(GGGS)_3$-linker is in yellow, vaccine peptide is in green, two epitopes are in pink. Different variants of vaccine peptide insertions are marked with numbers: 1: N-terminal; 2: C-terminal; 3: between 154 and 155 C-terminal amino acid residues, marked with red; 4: via polypeptide linker extension of C-terminus.

the CP sequence limits the peptide length and recombinant TMV (rTMV) recovery; however, the *Agrobacterium*-based transient system has allowed the development of an alternative method of rTMV particle generation [7]. *N. benthamiana* leaves coagroinjected with TMV and potato virus X-based vectors provide an accumulation of the nanoparticles, containing TMV RNA and potato virus X-encoded TMV CP C-terminal extension variants (Figure 4.3). In this approach, a 36-amino acid HER2/neu peptide binding the humanized monoclonal antibody, trastuzumab, was fused with the CP sequence via a flexible linker $(GGGGS)_3$. A rational design of the

Ad HER2/neu human epidermal growth factor receptor-2 that confers aggressiveness to breast cancers

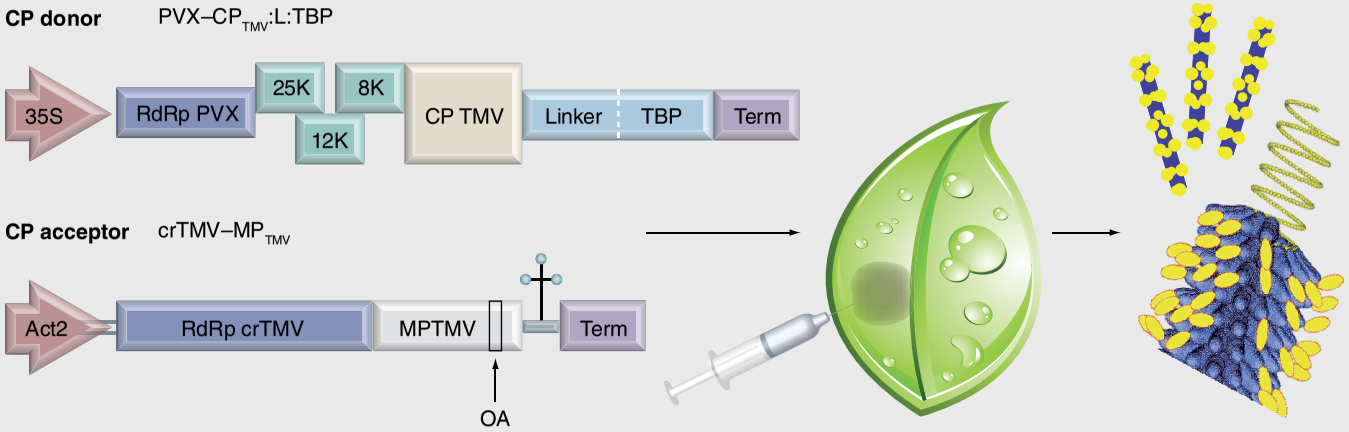
Trastuzumab a recombinant humanized monoclonal antibody that binds to the extracellular domain of HER2/neu, improving survival in patients with $HER2^+$ breast cancer

Table 4.1. Selected examples of vaccine peptides displayed by tobacco mosaic virus particles.

Epitope peptide display arrangement	Sites of foreign peptide insertion (Figure 4.2)	Inoculation method	Recovery (mg/g) fresh leaf weight	Ref.
8-aa mouse tumor T-cell epitope at the CP N-terminus	1	RNA transcript inoculation of tobacco	NI	[8]
12-aa malarial epitope at the C-terminus	2	RNA transcript inoculation of tobacco	NI	[9]
17-aa CRPV and ROPV epitope insertion at the CP C-terminal 154–155 position	3	RNA transcript inoculation of tobacco	NI	[10]
FMDV 28-aa F18 epitope inserted at the CP C-terminal 154–155 position	3	RNA transcript inoculation of tobacco	0.16–13.8	[6]
18-aa poliovirus VP3 and VP peptide insertion at the CP C-terminal 154–155 position	3	RNA transcript inoculation of tobacco	0.2	[11]
14- or 36-aa HER2/neu epitopes at the CP C-terminus via a flexible linker $(GGGGS)_3$	4	Agro-injection of two binary vectors: tobacco mosaic virus- and potato virus X-based	1.0	[7]

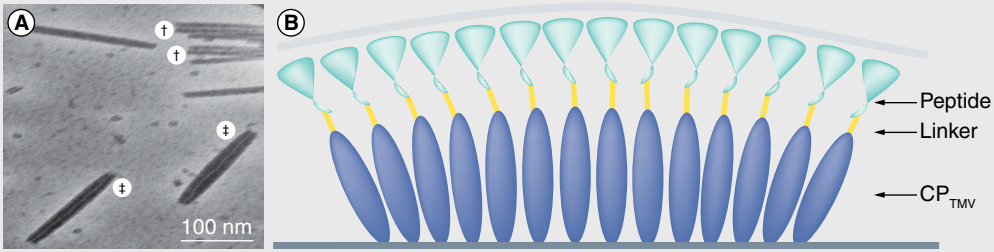
aa: Amino acid; CP: Coat protein; CRPV: Canine parvovirus; FMDV: Foot and mouth disease virus; NI: Not indicated; ROPV: Rabbit oral papillomavirus.

Figure 4.3. Recombinant virion production: potato virus X-based vector, coat protein donor and tobacco mosaic virus-based vector, and coat protein acceptor.



The agroinfiltration procedure is represented by the leaf and syringe and the depiction of the purified recombinant viral particles is in the rightmost position.
25K, 12K, 8K: Components of potato virus X triple gene block; 35S: Cauliflower mosaic virus 35S promoter; Act2: *Arabidopsis* actin 2 promoter; CP$_{TMV}$: Tobacco mosaic virus coat protein gene fused with vaccine peptide (trastuzumab binding peptide) via linker; MP: Movement protein gene; OA: Origin of virion assembly recognised by tobacco mosaic virus coat protein; PVX: Potato virus X; RdRp: RNA-dependent RNA polymerase gene; TBP: Trastuzumab binding peptide; Term: Terminator of transcription; TMV: Tobacco mosaic virus.

Figure 4.4. Recombinant tobacco mosaic virus particles.



(A) Transmission electron micrograph of purified recombinant viral particles. **(B)** 'Curl-brush' model of recombinant TMV surface: blue corresponds to coat protein part, yellow corresponds to linker, and green corresponds to the vaccine peptide. Curved line corresponds to the outer virion surface and straight line to the inner surface.
†Rod shaped particles.
‡Spindle-like virons.
CP: Coat protein; TMV: Tobacco mosaic virus.

amino acid-substituted trastuzumab-binding peptide (sTBP) that does not inhibit efficient rTMV assembly has been developed [7]. The physicochemical parameters of sTBP, including the charge and isoelectric point of the resultant fusion, should be maximally approximated to those of native CP. It has also been shown that only sTBP lacking cysteine provides efficient rTMV assembly. In addition, to increase rTMV production, putative *N*-glycosylation sites should be deleted or substituted in sTBP. Because TMV is not glycosylated and does not exploit the secretory pathway, the biochemical nature of the amino acid sequence in the context of the whole epitope is likely to provide the efficient assembly *per se*. Electron microscopic observations of the rTMV virions have revealed two types of particles. The first major type appeared to be typical rod-shaped virions that were similar to wild-type TMV; the second minor particle type appeared to be various irregular, spindle-shaped particles with uneven diameters **(Figure 4.4A)**. It is likely that the foreign peptide residues played interruptive roles in the structure of each single viral particle and affected the viral morphology, possibly due to microstructural alterations, as compared with the most stable TMV architecture. Moreover, the spindle-shaped particles might have been the result of linker and vaccine peptide 'curliness' on the surface of the rTMV particle, as shown in the scheme in **Figure 4.4B**.

Recombinant TMV particles with exposed sTBP have retained trastuzumab-binding capacity for at least 1 month following preparation and a comparison of the immunogenicity of rTMV and sTBP has shown that the TMV particles have high adjuvant properties.

Trastuzumab-binding peptides peptides of HER2/neu with vaccine and antitumor properties

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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Summary

- Tobacco mosaic virus (TMV) is an excellent biotechnological tool because viral particles are very stable and have high accumulation rates and immunogenicity.
- TMV is easy to purify from infected plants and may be used for vaccine development.
- A system based on an *Agrobacterium*-mediated codelivery into plant leaves of binary vectors encoding TMV RNA and a coat protein with a vaccine peptide in its C-terminal extension has been developed.
- For the efficient assembly of TMV, the physicochemical parameters of the vaccine peptide should be maximally approximated to those of the native coat protein.
- A flexible peptide linker between the coat protein and the vaccine peptide increases the efficiency of TMV assembly.
- Recombinant TMV particles with exposed trastuzumab binding peptide retain trastuzumab-binding capacity.

References

1 Ivanowski D. Concerning the mosaic disease of the tobacco plant. *Trans. J. Johnson.* In: *Phytopathological Classics Number 7.* American Phytopathological Society, MN, USA, 27–30 (1892 [1968]).

2 Beijerinck MW. Concerning a contagium vivum fluidum as cause of the spot disease of tobacco leaves. *Trans. J. Johnson.* In: *Phytopathological Classics, Number 7.* Johnson J (Ed.). American Phytopathological Society, MN, USA 33–52 (1898 [1968]).

3 Dorokhov YL, Ivanov PA, Novikov VK *et al.* Complete nucleotide sequence and genome organization of a tobamovirus infecting *cruciferae* plants. *FEBS Letters* 350, 5–8 (1994).

4 Marillonnet S, Giritch A, Gils M. *In planta* engineering of viral RNA replicons: efficient assembly by recombination of DNA modules delivered by *Agrobacterium*. *Proc. Natl Acad. Sci. USA* 101, 6852–6857 (2004).

5 Marillonnet S, Thoeringer C, Kandzia R *et al.* Systemic *Agrobacterium tumefaciens*-mediated transfection of viral replicons for efficient transient expression in plants. *Nat. Biotechnol.* 23, 718–723 (2005).

6 Li G, Jiang L, Li M *et al.* Morphology and stability changes of recombinant TMV particles caused by a cysteine residue in the foreign peptide fused to the coat protein. *J. Virol. Methods* 140, 212–217 (2007).

7 Frolova, OY, Petrunia IV, Komarova TV *et al.* Trastuzumab-binding peptide display by *Tobacco mosaic virus*. *Virology* 407, 7–13 (2010).

8 McCormick AA, Corbo T, Wykoff-Clary S *et al.* TMV-peptide fusion vaccines induce T-cell mediated immune responses and tumor protection in two murine models. *Vaccine* 24, 6414–6423 (2006).

9 Turpen TH, Reinl SJ, Charoenvit Y *et al.* Malarial epitopes expressed on the surface of recombinant tobacco mosaic virus. *Biotechnology (NY)* 13, 53–57 (1995).

10 Palmer KE, Benko A, Doucette SA *et al.* Protection of rabbits against cutaneous and mucosal papillomavirus infection using recombinant tobacco mosaic virus containing L2 capsid epitopes. *Vaccine* 24, 5516–5525 (2006).

11 Fujiyama K, Saejung W, Yanagihara I *et al. In planta* production of immunogenic poliovirus peptide using tobacco mosaic virus-based vector system. *J. Biosci. Bioeng.* 101, 398–402 (2006).