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Plant Production of Vaccine Against HPV: A New Perspectives

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1. Introduction

Infection by human papilloma virus (HPV) attracted attention in connection with cervical cancer in humans (zur Hausen, 1996). HPV type 16 alone accounts for approximately 50% of all cases of cervical cancer. The virus icosahedral capsid is composed of the L1 major and the L2 minor proteins. L1 alone has the capacity to self-assemble into virus-like particles (VLPs) without participation of L2 or other proteins. Because of similar immunogenicity compared to infectious virions, VLPs can be produced and used as a safe prophylactic vaccine against viral transmission of cervical cancer. During recent years two highly efficient VLP-based HPV vaccines (e.g. Gardasil, *Merck MSD* and Cervarix, *GlaxoSmithKline*) become available. For commercial production of vaccines and recombinant therapeutics, plants are often considered as a cost-effective alternative with several benefits. Firstly, production in plants can be easily scaled up in the case of acute demand for production and secondly, produced proteins are unlikely to be contaminated by human or animal pathogens, toxins and oncogenic sequences. Moreover, plants provide a convenient environment for protein expression and storage including the possibility of direct administration as edible vaccine if expressed in the appropriate plant tissue. In this article, we report recent promising advances in the production of prophylactic and therapeutic vaccines against HPV by expression of the relevant antigens in plants, and discuss future prospects for the use of such vaccines.

2. HPV vaccines

2.1 Structure of HPV capsid and neutralizing epitopes on its surface

Papilloma viruses (PVs) are small tissue specific double-stranded DNA tumor viruses, classified in the taxonomic family of *Papillomaviridae*. The Human Papilloma Viruses (HPVs) are phylogenetically closely related with similar biological properties among each other and with animal papillomaviruses that are host-specific to other vertebrates including amphibians, reptiles, birds and a variety of land and sea mammals. Animal PVs have been studied either as disease carrier and transmitters in animals or as models of human PV infection (Brandsma et al.; 1994; Campo 1997). Due to etiological connection with the high-rate mortality cervical cancer, the main attention is concentrated on the genital high-risk HPV types 16, 18, 33 and 58 as the leading cause of cancer (Munoz et al., 2003). The low-risk types 6 and 11 are associated with benign epithelial papillomas or warts that occur in 5–12% of normal women (Heim et al,

1995), however, HPV6 and 11 are the most commonly diagnosed to coinfect and comorbid in immunosuppressed individuals with malignant HPVs (Jay & Moscicki, 2000).

Papillomavirus infection induces type-specific immune response, directed mainly against the major capsid protein L1, rather than L2 minor protein, which also participates in the formation of the shell of native HPV particles. The viral capsid is primarily composed of 360 copies of L1 protein organized into 72 L1-pentamers (capsomeres) and associated with 12 or more copies of the minor L2 protein. When expressed in various recombinant system, L1 readily self-assembles, even in the absence of L2, into noninfectious virus-like particles (VLPs). VLPs are also organized into 72 capsomeres of L1 protein (Fig. 1C) and are immunologically indistinguishable from the native virions (Fig. 1B). The 504-residue of the L1 protein chain contains 12 β-strands, 6 loops, and 5 α-helices that form "jelly roll" β-sandwich (Fig. 1D).

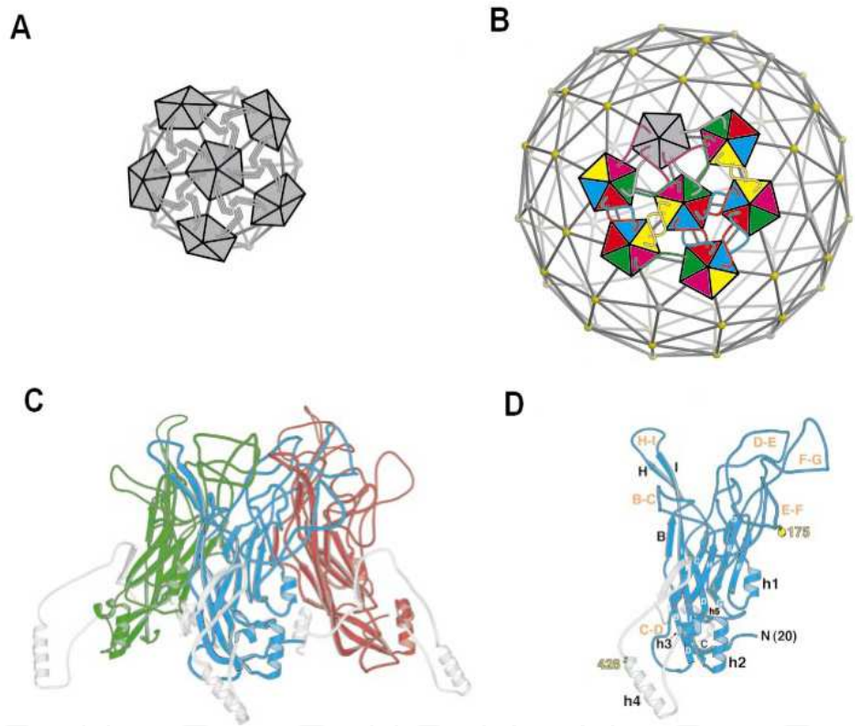


Fig. 1. Structure of the HPV capsid. (A) Small (T=1) VLPs derived from HPV-L1; (B) Full-size (T=7) Papillomavirus particles; (C) HPV16 capsomere (L1-pentamer) in the conformation found in small VLPs (Chen et al., 2000). Three subunits are shown in green, blue and red. The C'-terminal arms are in gray, to indicate that these portions of the subunit rearrange when L1-pentamers assemble into virions or into full-sized capsids; (D) The 3D structure of human papillomavirus 16 L1 monomer (HPV16L1). Secondary structural elements are labeled, with letters *B–J* for β-strands and h1–h5 for the 5 α-helices. Loops between strands are labeled *B–C*, *C–D*, etc. The first and the last residues are marked N (20) and C (474), respectively. The two cysteines that participate in the interpentamer disulphide bonding within the virion or in the virion-sized particles are shown in yellow, together with their residue numbers, 175 and 428. (Modis et al., 2002)

Some residues in the L1 protein, such as Asp202, Cys175, and Cys428 of HPV16 L1, are very important for VLP formation (Slupetzky et al., 2001), however some residues at the C'-

terminus can be truncated and replaced with heterologous epitopes or short polypeptides up to 60 amino acids without disrupting the assembly of VLPs (Paintsil et al., 1996; Müller et al., 1997; Paz De la Rosa et al., 2009). These chimeric VLPs (cVLPs) can induce strong immune responses against not only the inserted epitopes or polypeptides, but also the VLP shell (Freyschmidt et al., 2004; Varsani et al., 2003a; Xu et al., 2006). Experiments *in vitro* showed that a short N'-terminal segment of the L1 polypeptide chain acts as a switch between T=7 (72 L1-pentamer) and T=1 (12 L1-pentamer) VLP assembly (Fig. 1B) (Chen et al., 2000).

Structural analysis has revealed that BC, EF, FG, HI and DE hyper variable loops of L1 (Fig. 1D) are exposed on the outer surface of the L1-pentamer and form a broad pocket, which participate in receptor interaction. The rim of the pentamer pocket is extremely variable contrary to its floor. With a few exceptions, all HPV-neutralizing monoclonal antibodies analyzed so far are type-specific and recognize conformational epitopes within these surface-exposed hyper variable loops (Pastrana et al., 2004; Fleury et al., 2006). HPV16 and HPV11 VLPs epitopes recognized by neutralizing mAbs are shown on Fig. 2 (Roden et al., 1997a; Ludmerer et al., 1997).

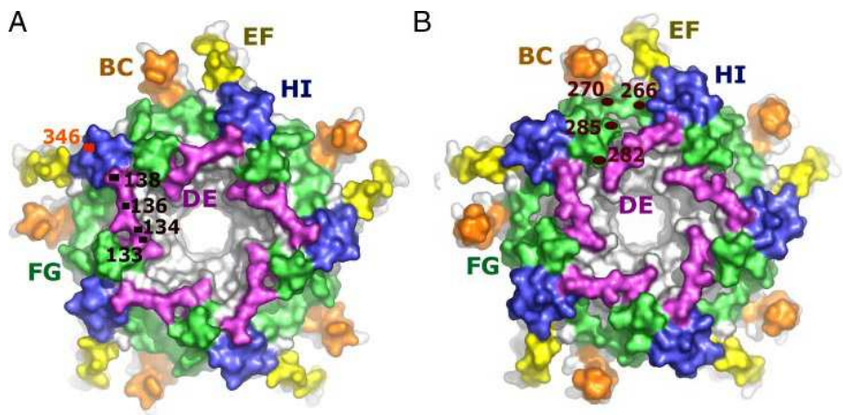


Fig. 2. Surface of the HPV 11 and 16 L1 pentamers. The surface loops are colored differently on the surface: BC (orange), DE (violet), EF (yellow), FG (green), and HI (slate). (A) HPV11; (B) HPV16. Neutralizing epitopes are schematically indicated on pentamers. Black squares, H11.F1 and H11.G5; orange square, H11.H3; red ellipse, H16.V5 and H16.E70. (Bishop et al., 2007)

2.2 HPV vaccines of the first generation. The need for second-generation vaccines

The first applied strategy for HPV prophylactic vaccination aimed on induction of neutralizing antibodies against L1 capsid proteins. Currently two vaccines composed of HPV-L1 self assembled into VLPs have been developed and are commercially available, a Glaxo-Smith Kline bivalent vaccine Cervarix and a Merck, Sharp and Dohme quadrivalent vaccine Gardasil (also marketed as Gardisil or Silgard). Bivalent vaccine Cervarix protects primarily against HPV 16 and 18 that are produced separately using a recombinant Baculovirus expression system. Purified VLPs of each HPV type are formulated with the AS04 adjuvant system composed of aluminium hydroxide and 3-*O*-desacyl-4.-monophosphoryl lipid A (MPL). Cervarix is stored as a sterile turbid liquid suspension for intramuscular injection at 2-8°C with a proposed shelf life 3 years. Gardasil is combination of *Saccharomyces cerevisce* produced HPV 16, 18, 6 and 11 VLPs and has amorphous

aluminum hydroxy-phosphate sulfate (AAHS) as an adjuvant. Administrations of vaccine to HPV naive women have demonstrated almost complete protection against infection by the targeted HPV types. Number of medical trials confirmed the safety of currently used vaccines, their efficiency to induce immune response was equal or even higher than that observed during a natural infection and to maintain protection for 5 and 7.3 years in the case of Gardasil and Cervarix, respectively. (McCormack & Joura, 2010; Schwarz, 2009). The induction of HPV neutralizing antibody reaches maximum titers at month 7 after the first vaccination, i.e. 1 month after administration of the last, third dose. Then the titer decline until month 24 and remain stable thereafter (Dillner et al., 2007). Interestingly, the immune response to the Gardasil, the tetravalent vaccine, inversely correlates with the age. The induction of neutralizing antibody is higher in males and females aged 10 to 15 than in those of an age group 16 to 23 year (Villa et al., 2005). For sexually active women in the general population the efficiency of vaccination is expected to be much lower. Moreover, total period of protection afforded by vaccination is not yet known (Wright et al., 2006).

Along with the questions who should be vaccinated and at what age the vaccination is the most effective (Villa, 2011), issues related to vaccine formulation, production and administration have to be also adequately resolved (Schiller & Nardelli-Haefliger, 2006). Firstly, multispecific VLPs based vaccines are expensive to manufacture, since they are produced in eukaryotic cell culture and extensively purified. Both commercially available vaccines require 3 intramuscular injections over a 6 months course to achieve prophylaxis, and the direct vaccine cost excluding administration and medical visits is about USD 375 per recipient in the United States (Armstrong, 2010), reflecting also costly cold chain handling, distribution and storage of a vaccine. More to it, the protection with current vaccines is predominately type specific, and so they are not expected to protect against the almost 30% of cervical cancers that are HPV16 and 18 independent. Last, but not least, therapeutic activity against external genital lesions has not been reported (Villa, 2011).

Suitable vaccine formulations ensuring VLPs stability in liquid were established for both Gardasil (Shank-Retzlaff et al., 2006) and Cervarix (Le Tallec et al., 2009) vaccines. However during production fibrous aggregates of VLPs were occasionally observed. This is why marketed solutions of concentrated VLPs are protected against aggregation by high concentrations of salt. Many other factors including excipients maintaining pH, storage stability, temperature and time effectively influence VLPs. For example at high pH and low salt concentration, VLPs disassemble into capsomeres with weaker immune response than VLPs (Thönes et al., 2008) and denatured L1 protein does not induce any virus neutralizing antibody response (Shinje et al., 1991). Stabilization of protein antigens against aggregation and degradation in solution is important for antigen purification as well as vaccine formulation. Lyophilized VLPs might be an alternative to aqueous droplets for mucosal delivery in a powder formulation of a vaccine (Schiller & Nardelli-Haefliger, 2006; Gerber et al., 2001). Papillomavirus virions are resistant to desiccation and retain their native conformation after freeze-drying (Roden et. al, 1997b; Šmídková et al., 2010). However, both commercial vaccines contain an alum adjuvant, which during freeze-drying extensively coagulate into gel-like consistency, the state suspected to inhibit the release of antigen upon rehydration (Maa et al., 2006). The loss of efficient immune induction has been reported after freezing or freeze-drying of VLPs in aqueous solutions (Shinje et. al, 1991). The use of non-ionic stabilizers, such as methylcellulose (Corbett et al., 2010) or polysorbate PS80 (Shi et al., 2005) helps to avoid this effect. Another solution could be the use of another, physically and chemically more suitable adjuvant(s), but this strategy would further add to the cost of vaccine

and principally could raise safety concerns. This is why convenient dry formulation, meaning at least longer shelf life at higher temperatures for marketing purposes is currently missing. The examples of the alternative adjuvants to alum are discussed in Section 3.

Several alternative methods of vaccine needle delivery have been developed. The tattoo delivery of DNA has been found as a cost-effective method that may be used in laboratory conditions when more rapid and more robust immune responses are required (Pokorná et al., 2009). The second method, a novel dry-coated densely packed Macroflux® microprojections array skin patch, was established as an alternate delivery system to intramuscular injection for delivering an alum adjuvanted vaccine Gardasil (Shi et al., 2005).

Besides the effort to improve the formulation, storing properties and methods of vaccine delivery, the first generation of HPV prophylactic vaccines based on IM delivery of HPV antigens of the recombinant VLPs reached its limits with two vaccines produced in baculovirus and yeast cell culture on the market. In parallel with development of these vaccines there were successful attempts to produce recombinant VLPs also in various plant expression systems (see Section 2.4). Plant expression of HPV vaccines pursues several objectives as the cost efficiency, production of uncontaminated safe product, scale up and potential edible vaccine format. Currently HPV VLPs are readily produced in several plant systems with newly developed technologies for industrial large-scale transient production that can successfully compete with current production of L1 based VLPs vaccines. Nevertheless the cost of clinical trials, of approval and implementation of new technologies is so inhibitory high that there will be hardly any HPV vaccine of the first generation produced in plants that will reach the market. More realistic expectation is that developed plant technologies will compete with established procedures for production of new, improved generation of vaccines, when their advantages will surplus currently marketed vaccines and expected market success will justify the cost of clinical trials and production.

2.3 Vaccines of the second generation

2.3.1 L2 based vaccines

The limitation of current vaccines is that neutralizing antibodies induced by immune response to L1 based VLPs are type-restricted (Wakabayashi et al., 2002). Addition of other HPV types VLPs to the existing vaccines would be viable approach only in the case of a small increase in the overall cost of the vaccination scheme. In contrast to L1, Pastrana et al. (2005) showed in *in vitro* assays that antigen determinant present on the N'-end of L2 coat protein can induce broad range of cross neutralizing antibodies in mouse and rabbit sera. These results raise the possibility that a monovalent vaccine could protect against a broad range of genital HPV types. Unfortunately, neutralizing antibody titers against the papillomavirus type from which the L2 vaccine was derived were generally higher than the titers against heterologous types and lower than those induced by L1 VLPs (Pastrana et al., 2005; Roden et al., 2000). This can be avoided by construction of concatenated multiple L2 fusion proteins derived from known cross-protective epitopes of several divergent HPV types. These fusion proteins, consisting of L2 epitopes of 3-22 HPV types, were able to induce high neutralizing antibody titers against all heterologous HPVs tested at a level comparable to that induced by L1 VLPs. In addition, L2 polypeptides have the advantage that they could be produced in *E. coli*, and therefore manufacturing would be easier and cheaper in comparison to production of VLPs. The most promising approach of the non-VLPs second generation of HPV vaccines includes L1 capsomeres and L2 protein (Stanley, 2010).

2.3.2 Therapeutical vaccines

The therapeutic vaccines reduce or eradicate existing disease or infections by targeting cells expressing tumor-associated or tumor-specific antigens on their surface (Ma et al., 2010). There are many different types of therapeutic vaccine candidates based on viral gene peptides and proteins (Xie et al., 2011; Kenter et al., 2008; Melief et al., 2007; Fiander et al., 2006), DNA (Alvarez-Salas et al.; 2008, Sheets et al., 2003) and various viral and bacterial vectors (Brandsma et al, 2009, Davison et al, 2003). They all aim to induce specific cell-mediated immunity and in most cases the targets are the E6 and E7 proteins. Whereas L1 and L2 are expressed only in terminally differentiated keratinocytes, E6 and E7 are constitutively expressed at all layers of epithelium-infected cells (Fig. 3).

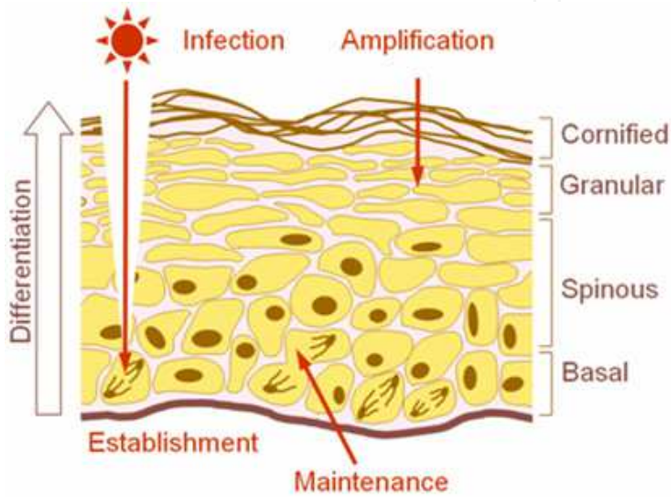


Fig. 3. Cervical stratified squamous epithelial cell architecture and the expression of HPV proteins after infection. Daughter cells of epithelial stem cells divide along the basement membrane and then mature vertically through the epithelium without further division. After introduction of HPV into stem cells in the basal layer of the epithelium, expression of viral non-structural proteins E1-E7 occurs. Under the regulation of these proteins, the dividing-cell population expands vertically and epithelial cell differentiation is delayed and is less complete. Viral proteins are expressed sequentially with differentiation as shown, and mature virions are produced only in the most superficial layers of the epithelium. Intraepithelial antigen-presenting cells (APCs) are depleted in the HPV-infected epithelium. (http://www.ircm.qc.ca/LARECHERCHE/axes/Biologie/Virologie/Pages/Projets.aspx)

E6 and E7 bind p53 and pRB human tumor suppressor genes (Duensing et al., 2000). These oncoproteins are involved in the malignant transformation of HPV-infected cells and are thought to be required for continued tumor growth. They are the primary targets of therapeutic vaccines, most of which have been designed to treat later stages of the disease. The E1 and E2 proteins are necessary for HPV replication within the cell before the virus is integrated into the host DNA (Doorbar et al., 1991). Because E1 and E2 are expressed already early in the progress of at HPV infection and at higher levels than E6 and E7, they may be the best targets for a therapeutic vaccine designed to treat early stages of the disease, such as low-grade dysplasia (Carvajal et al., 2007). Many candidate vaccines with therapeutic potential are currently tested in ongoing trials; however, there is low expectation

that any of the current therapeutic vaccines will have a substantial public health impact in the near future (Ma et al., 2010).

2.4 Vaccine production in plants

2.4.1 Posttranslational modification of the therapeutic proteins in plants

Therapeutic recombinant proteins are produced in many hosts from prokaryotes to human cells. When the protein of interest is of eukaryotic origin, one of the production objectives besides yield, solubility and stability is a posttranslational modifications (PTM) required for structural integrity and biological activity of the protein. Microbial expression systems are generally used for expression of simple proteins, because, PTM, including signal peptide cleavage, propeptide processing, protein folding, disulfide bond formation and glycosylation, might not be achieved in prokaryotes. Contrary to prokaryotes, plants are capable of PTM as other higher eukaryotes for safe and low cost production biologically active proteins (Dieryck et al., 1997; Ma et al., 1995). The correct folding and assembly of plant-produced antibody molecules, which requires interactions with several chaperones and with processing and glycosylation enzymes, illustrates that most co- and posttranslational events are similar in plants and mammals (Table 1).

Protein glycosylation is assumed as the most important PTM with significant effects on protein folding, conformation, distribution, stability and activity. In plant cells, as in other eukaryotic cells, N-glycosylation starts during co-translation in ER, when an oligosaccharide precursor is added to Asn residues that is constituent of the N-glycosylation-specific sequences Asn-X-Ser/Thr. The differences in the maturation of plant and mammalian N-glycans appear during the late processing in Golgi apparatus, when core alpha(1,6)-linked fucose residues and terminal sialic acid are attached in mammals, whereas beta(1,2)-xylose and core alpha(1,3)-fucose residues in plants (Fig.4).

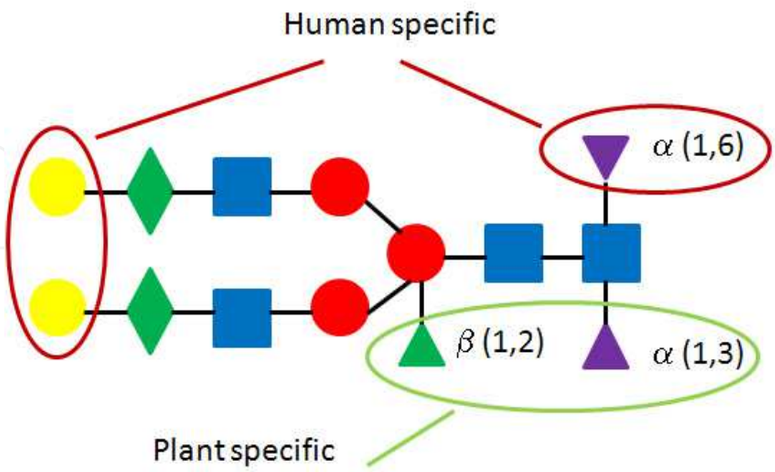


Fig. 4. N-glycan structures in human and plant. N-glycosylation patterns processed in plant cells differ from those of mammal

Posttranslational modification	Location of the reaction in animal cell	Identification in plants and location
Glycosylation		
O-glycosylation	Secretory pathway, nucleus and cytosol	Yes. Sugar addition on Hyp, Ser, Thr
N-glycosylation	Secretory pathway	Yes. Minor differences in modification
Glycation		Yes
Proteoglycan	Secretory pathway	Yes
Attachment of fatty acids		
S-acylation	Cytosol	Yes
N-myristoylation	Cytosol	Yes
Prenylation	Cytosol	Yes
Glypiation	Cytosol	Yes
Cholesterol link	Cytosol	Not identified.
Attachment of ions		
Phosphorylation	Cytosol, secretory pathway	Yes. No mannose-6-phosphate in plant N-glycans
Sulfatation	Secretory pathway	Not identified in secretory pathway
Gamma-carboxylation	ER	Not identified.
Proteolysis		
Cleavage of signal peptide	ER	Yes. ER
Cleavage of propeptide	ER/Golgi	Yes. ER, Golgi, vacuole
Other posttranslational modifications		
Hydroxylation	ER	Yes. ER, Golgi
Cross-linking modifications	ER	Yes. ER, cytosol
Acetylation	Secretory pathway and cytosol	Yes. Function not yet understood in plants
Oligomerization	ER	Yes. ER
Selenoprotein	Cytosol	Not identified in higher plants, described in *C. reinhardtii*
Deamidation	Intra- and extracellular	Yes
Oxidation	Intra- and extracellular	Yes

Table 1. Protein modifications in plant and animal cells. (Gomord & Faye, 2004, modified)

Plant specific residues were described to be constituents of the glycol-epitopes of some plant allergens, showing IgE binding and causing mediator release by human basofils (van Ree et al., 2000). Moreover, the injection of a plant glycoproteins or plant-made antibodies containing plant-specific N-glycans was found to elicit production of antibodies specific for beta(1,2)-xylose and alpha(1,3)-fucose-containing glyco-epitopes in most laboratory mammals and non-allergic human blood donors. Their presence may induce a rapid immune clearance of plant-glycosylated therapeutics from the blood steam (Bardor et al., 2003). One strategy to prevent the addition of immunogenic glycans is to store therapeutic proteins carrying KDEL signal within ER (Ko et al., 2003). Second strategy is based on the inhibition of Golgi glycosyltransferases of plants. The moss *Physcomitrella patens* is the only known plant with high frequency of homologous recombination, thus allowing relatively easy knockouting of target genes. The knockout of alpha(1,3)-fucosyltransferase and beta(1,2)-xylosyltransferase genes in the moss *Physcomitrella patens* prevents the production of plant-specific glyco-epitopes without effecting the secretion of the protein (Koprivova et al., 2004). Third attractive strategy to "humanize" plant N-glycans is expressing of mammalian glycosyltransferases in plants. Like N-glycosylation, O-glycosylation is important for protein function. Surprisingly, little attention is paid so far to O-glycosylation status of plant produced therapeutic proteins.

2.4.2 L1 based prophylactic vaccines and the yields of L1 produced in different plant systems

L1 when expressed in plants readily assemble into VLPs indistinguishable in size from baculovirus expressed VLPs in insect cells (Fig. 5). Sucrose sedimentation analysis also showed that there is a large amount of not, or only partially assembled molecules, presumably capsomeres (fractions 18–24), as well as other larger aggregates (fractions 1–8) when compared to the insect cell-produced protein (Maclean et al., 2007). L1VLPs can be produced either in transgenic plants stably transformed with an expression cassette or transiently using one of several available plant-virus derived expression systems. Initially published yields of L1 expression was low, in a range of 1% of the total soluble protein, which is far lower than industrial demand of more than 5% (Rybicky, 2010). For example, Warzecha et al. (2003) obtained approximately 20 ng HPV-11 L1 per g in transgenic potato tubers; Varsani et al., 2003b, 2006 obtained 4 ng HPV-16 L1 per g of leaf tissue in transgenic tobacco and approximately 40 ng per g of *Nicotiana benthamiana* leaves transiently transformed with a tobacco mosaic virus vector and Kohl et al. (2006) and Liu et al. (2005) achieved approximate yield of L1 at the range 0.05% TSP in transgenic tobacco.

Nevertheless, during passed years, necessary steps to improve *L1* gene expression were recognized and applied. Firstly it was removal of the carboxy-terminal nuclear localization signal sequence (NLS) of L1 that has been shown to enhance expression in transgenic plants. Moreover, the results indicated that full-length L1 is localized essentially entirely within the nucleus (Fig. 6A), whereas cells that express truncated form of L1 in a diffuse pattern within entire cell (Fig. 6B.) (Warzecha et al., 2003). Transient expression of full-length L1 protein in cytoplasm of tobacco leaf cells after agroinfection was described by Šmídková et al. (2010) (Fig. 6C).

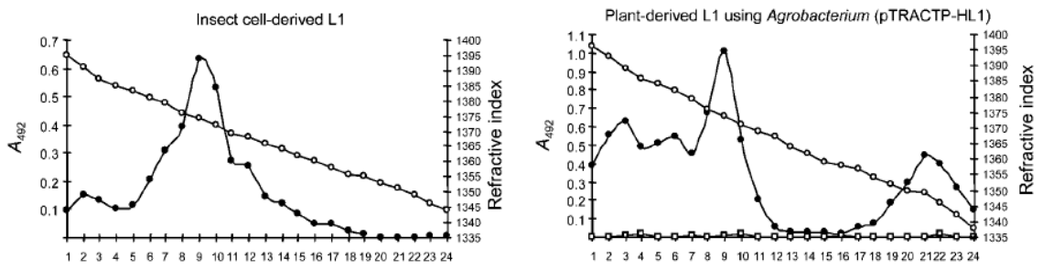


Fig. 5. Sedimentation analysis of transiently expressed HPV-16 L1 protein isolated from *Nicotiana benthamiana* plants (left panel) and insect cells (right panel) in sucrose gradient. The concentration of L1 in fractions was estimated by capture ELISA (closed circles); open squares – ELISA analysis of control, no expressing plants; open circles and right axis - refractive index. Fraction 1 corresponds to the bottom of the centrifuge tube (Maclean et al., 2007).

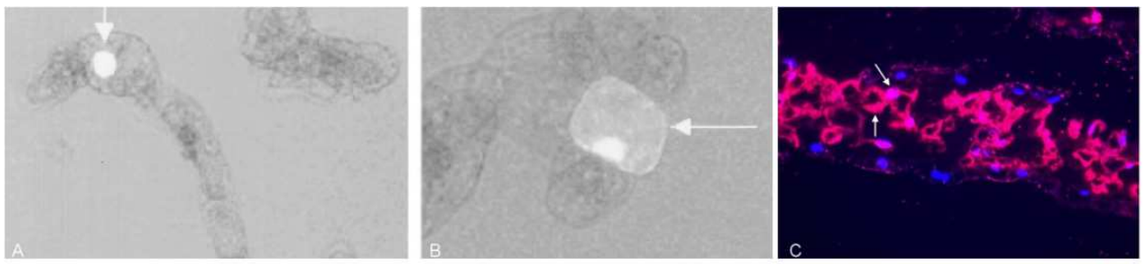


Fig. 6. Expression of HPV L1 proteins in tobacco cells. Transgenic tobacco expressing either full-length (A) or truncated (B) L1 coding sequence fused in frame at the carboxy terminus of GFP. Fluorescence appears as bright areas (Warzecha et al., 2003) (C) Localization of L1 in cryosections of *Nicotiana benthamiana* leaves agroinfected with plant TRV viral vector expressing optimized *L1h* gene (pTVL1h). The protein was detected by immunofluorescence microscopy at 400X magnification. Nuclei were counterstained with DAPI (Šmídková et al., 2010).

Biemelt et al. (2003) after failure to express L1 in transgenic potatos, changed amino-acid codon usage of *L1* gene to that of potato (*L1p*) and of canonic human cells (*L1h*). Despite the presence of codons rarely used by plant cells and for plant genes atypical high GC content, expression of *L1h* led to high accumulation of L1 protein in transgenic plants, even higher than expression of *L1p*. The effect of increased GC content on expression efficiency of *L1* in plants has been confirmed by several groups (Maclean et al., 2007; Šmídková et al., 2010), nevertheless the published yields of L1 differ significantly one from each other, depending on the plant expression system used. For example the change of tobacco cultivar used for transgenic expression from *Nicotiana tabacum* cv. Xanthi to cv. SR1 allowed a 100-fold increase in expression of the native *L1* from viral isolate (Rybicky, 2010). The transient expression of the same *L1* using TMV plant viral expression vector resulted in further, one order of magnitude, increase of L1 over the expression in transgenic tobacco (Varsani et al., 2006). A strategy of optimization procedure for L1 transient expression described recently Šmídková et al. (2010). The results are summarized on Fig. 7, depicting A) The course of L1 expression from original virus isolate sequence (41% GC) and *L1p* sequences optimized for

Solanace plants expression (39% GC) and from canonic human cell optimized sequence *L1h* (61% GC); B) In three plants: tomato and *Nicotiana benhamiana* and *Nicotiana tabacum* tobaccos; C) In two plant viral expression vectors: PVX and TRV and by D) Two transformation methods: agroinfection (method in which a virus infects a host as a part of T-DNA of Ti plasmid carried by *Agrobacterium tumefaciens*) and *Agrobacterium* mediated transfer of expression cassette into cells after infiltration of leaves. The optimization of L1h transient expressed from pTRV vector after *Agrobacterium* infiltration of tomato host plants yielded 45 mg of VLPs per kilo of fresh leaves, the yield that is close to industrial acceptable level.

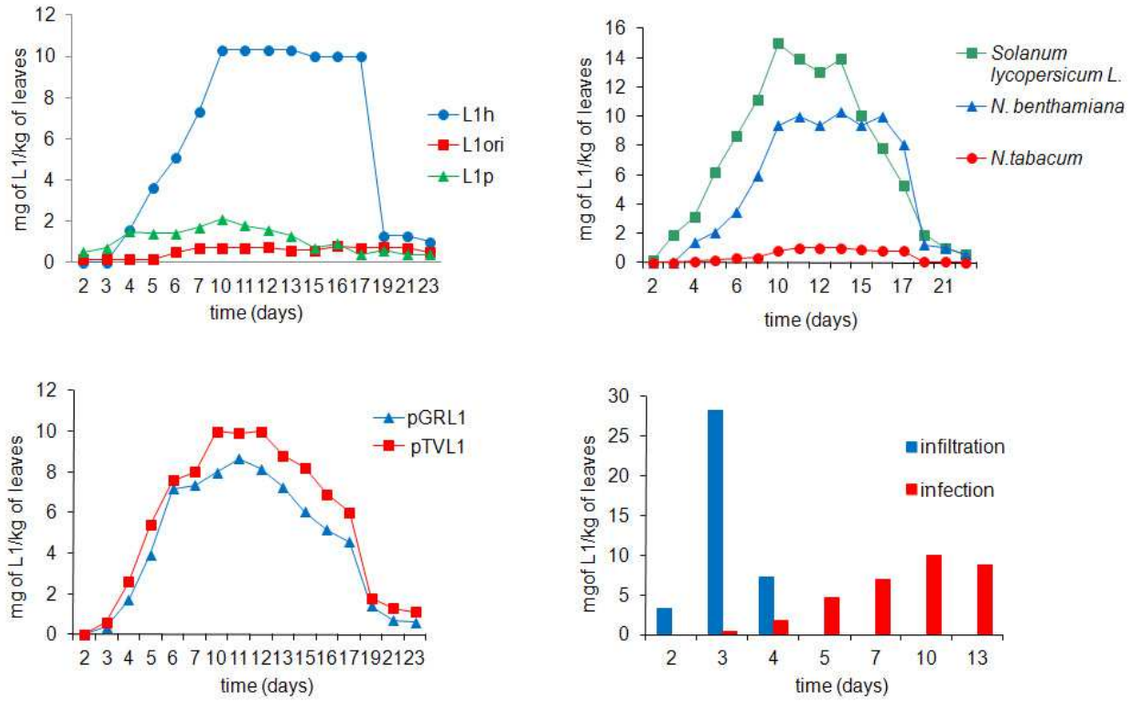


Fig. 7. Time course of the L1 transient expression. (A) Expression of original native HPV *L1* gene sequence *L1ori* and sequences optimized for expression either in plants *L1p* or in mammalian cells *L1h* from plant TRV-based vector pTV00; (B) Expression of *L1h* from pTV00 vector in leaves of *Nicotiana benthamiana, Nicotiana tabacum* and *Solanum lycopersicum L.* (tomato); (C) Comparison of the expression of *L1h* gene from plant tobamo virus (TRV) based expression vector pTV00 and from potato virus X (PVX) based vector pGR106; (D) The yield of L1protein reached by viral agroinfection or by *Agrobacterium* mediated transfer of expression cassette into leaf cells of *Nicotiana bentamiana* (Šmídková et al., 2010).

Nevertheless the highest yield (3 g/ kg fresh leaves; 24% TSP) of plant-produced L1 ever was achieved by Fernández-San Millán et al. (2008) when expressing unmodified *L1* sequence of primary HPV16 virus isolate from expression cassette stably integrated in tobacco chloroplasts genome. Expression in plant chloroplasts is an emerging system when compared to nuclear transformation. Plastid genome engineering offers many advantages over nuclear genome, including targeted recombination based integration, high levels of transgene expression due to high copy number, absence of epigenetic effects, transgene

containment via maternal inheritance and multi-gene expression in a single transformation event (Chebolu and Daniell, 2009). Recently the expressions of transgenes in plastids as high as 70% and 72% of total leaf protein was reported by Oey et al. (2009) and Ruhlman et al. (2010), respectively.

Results of L1 expression in various plant systems up to date are summarized in Table2.

Antigen	Production system and yield	Efficacy data
HPV-16 L1	*Agrobacterium*-transformed *Nicotiana tabacum* cv. Xanthi plants Assembled in VLPs 4 µg/kg ww	Weakly immunogenic in rabbit
HPV-11 L1	Transgenic potato tubers Assembled in VLPs 20 µg/kg	Weakly immunogenic in orally vaccinated mice
HPV-16 L1	Transgenic potato tubers Assembled in VLPs 12 mg/kg	Weakly immunogenic in orally vaccinated mice
HPV-16 L1	Transgenic tobacco plants Assembled in VLPs 20 mg/kg ww	Highly immunogenic in mice injected with purified product
HPV-16 L1	Protein expressed in *Nicotiana benthamiana* by TMV-derived vector 40 µg/kg wet leaves	ND
HPV-11 L1	Transgenic *N. tabacum* 2 mg/kg ww Transgenic *Arabidopsis thaliana* 12 mg/kg ww *N. benthamiana* via rTMV 10 mg/kg ww	ND
HPV-16 L1	Agroinfiltrated *N. benthamiana*, human codon usage-optimized gene; protein targeted to chloroplasts, assembled in VLPs 500 mg/kg ww	Antibodies elicited in mice by injection of crudely purified extracts neutralized HPV-16 pseudovirion transfection of HEK293TT cells
HPV-16 L1-Rubisco/ ATPase peptide fusion	Protein produced in chloroplasts of transplantomic tobacco plants from native or chloroplast-optimized genes 60 mg/kg ww	ND
HPV-16 L1	Protein produced from unmodified genes in chloroplasts of transplantomic tobacco plants 3 g/kg ww	Mice injected intraperitoneally with partially purified VLPs with Freund's or aluminium hydroxide adjuvants produced neutralizing antibodies

Table 2. Plant-derived HPV antigens for the development of prophylactic vaccines. HPV: Human papillomavirus; ND: No data; PVA: Potato virus A; PVX: Potato virus X; rTMV: Recombinant tobacco mosaic virus; TMV: Tobacco mosaic virus; VLP: Virus-like particle; ww: Wet weight. (Giorgi et al., 2010)

2.4.3 The structure and stability of plant derived L1

The assembly of VLPs in plants after transient (Fig. 8 A, C) or stable (Fig. 8D) L1 expression was confirmed by electron microscopy of leaf crude extracts.

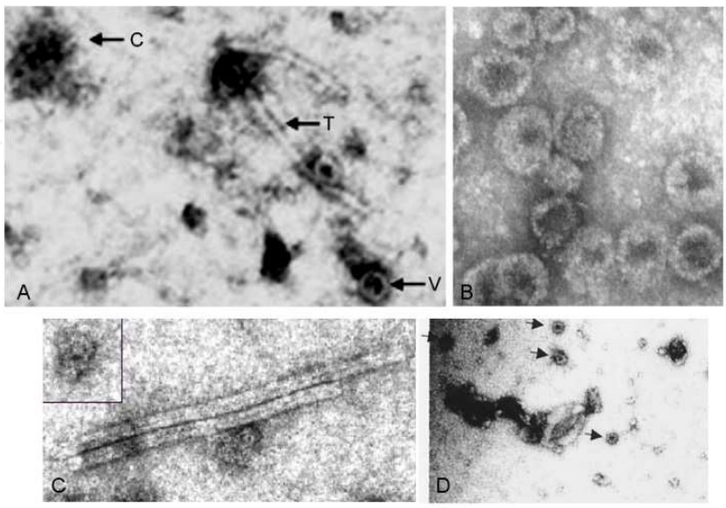


Fig. 8. Electron microscope images of uranyl acetate negatively stained HPV VLPs expressed in various systems: (A) Extracts prepared from freeze-dried leaves of *Nicotiana benthamiana* transiently expressing L1. V - HPV16L1 55-nm VLPs particles, C - HPV16 L1 capsomeres, T - flexible rods of plant TRV virus (Šmídková et al., 2010); (B) CsCl-purified baculovirus expressed VLPs in insect cells; (C) Crude extracts from top leaves of *Nicotiana benthamiana* expressing HPV VLPs from plant TMV virus. Two rods of TMV are shown together with VLPs (Varsani et al., 2006); (D) CsCl-purified VLPs from transgenic potato plants. VLPs have band density 1.32g/ml (Biemelt et al., 2003).

The structure of VLPs is not stable upon freezing and thawing, but plant expressed VLPs retain their structure during freeze-drying in both, the plant extracts and the plant tissue Fig. 8 and 9 (Maclean et al, 2007).

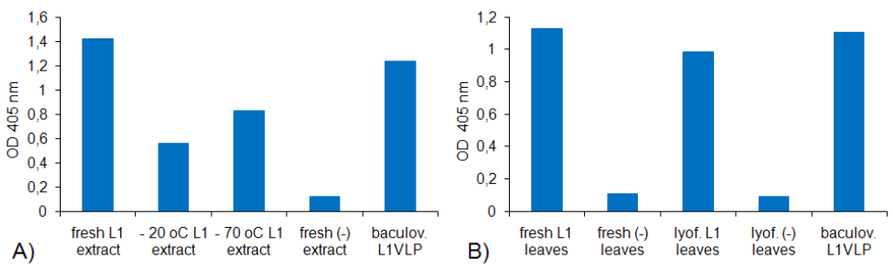


Fig. 9. Stability of L1 VLPs transiently expressed from pTV00 vector in *Nicotiana benthamiana* leaves: (A) after freezing and thawing extract from fresh leaves. Approximately 50% of VLPs loses 3D structure when extract is stored at -20°C or –70°C, respectively; (B) Upon freeze-drying of leaves and extraction cycle. More than 90 % of L1 retains conformation in extracts from freeze-dried leaves (*third column*) in comparison to extract prepared from fresh leaves (first column). Controls are extracts from leaves prepared the same way, but from plants inoculated with an empty pTV00 vector.

2.4.4 Immunogenicity of plant derived L1

The first report on production of HPV VLPs in plants and testing of their immunogenicity appeared in literature in 2003. Varsani et al. (2003b) was the first to express full-length native *HPV-16 L1* gene in transgenic *Nicotiana tabacum* cv. Xanthi. This plant-produced protein assembled into recognizable VLPs and was immunogenic, when injected into rabbits with Freund's incomplete adjuvant. Since then, several groups has observed induction of specific antibodies after subcutaneous injection of either purified plant-derived HPV16 L1 VLPs (Biemelt et al., 2003) or of the crude extract from the tobacco leaves expressing HPV16 L1 protein (Fig. 11A) (Maclean et al., 2007; Šmídková et al., 2010). Despite the fact that in plant extracts majority of expressed L1 protein was present as capsomeres besides VLPs Fig. 8A, the extracts were highly immunogenic without any additional adjuvant (Maclean et al., 2007, Šmídková et al., 2010). The antibodies induced by immunization with plant extract depicted on Fig. 8A preferentially recognized fully assembled L1 VLPs (Fig. 12A) and neutralized in vitro HPV16 virions (Fig. 12B). Plant expressed L1 in crude extract also induces CTL (Fig. 11B).

These findings suggests that principal antigenic determinant is either entire VLP or 3D structure specific for fully assembled VLPs and these antigens are present in crude extracts from plants transiently expressing L1 in enough quantity to elicit immune response equal or higher than purified VLPs from insect cells. Moreover immunization with plant L1 crude extracts induce cellular responses characteristic for active vaccine (Šmídková et al., 2010).

Mucosal delivery has several advantages over needle administration. Immune response is best achieved by direct application of a vaccine to mucosal surfaces and in addition mucosal application of a vaccine can also induce humoral, cell-mediated and systemic immune responses (Rigano & Walmsley, 2005). HPV VLPs are immunogenic when administered orally and stable in the environment of the gastrointestinal tract. Rose et al (1999) and Gerber et al. (2001) reported that as little as 1-10 micrograms are sufficient to induce high titers of L1 specific antibodies after oral application when administered with LT or CpG DNA adjuvants. Besides VLPs also capsomeres (L1-pentamers) and T=1 particles (12 L1-pentamers) depicted on Fig. 10 were investigated for oral immunogenicity in mice. Mutated L1 gene (L1_2xCysM) with two cysteines replaced by serines was used to generate capsomeres and T=1 particles. Compared to capsomeres, VLPs induced higher titers of neutralizing and IgA secreted antibodies, while cytotoxic T cell responses was comparable. The induction of secreted IgA antibodies was observed after oral but not after subcutaneous immunization (Thönes & Müller, 2007).

The concept of using tissue of plants expressing vaccine antigens as an edible vaccines attracted already a lot of attention and is still of special interest. A number of clinical studies demonstrated the induction of specific antibodies after oral immunization using crude plant material containing, for example, hepatitis B or Norwalk virus antigens (Lal et al., 2007). Likewise, oral immunization using crude potato tubers expressing L1 protein can induce specific antibody (Warzecha et al., 2003, Biemelt et al., 2003). Moreover, HPV L1-E6/E7 based chimeric VLPs have been successfully expressed in tomato fruits, which were able to elicit humoral and cytotoxic T-cell activity in mice (Paz De la Rosa et al., 2009).

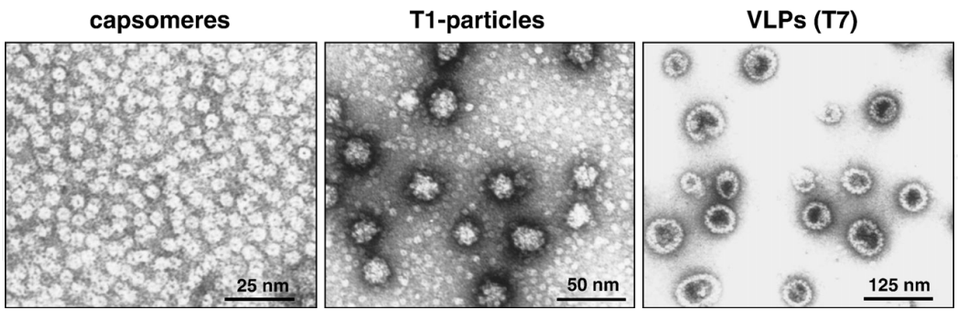


Fig. 10. Analysis of different L1 assembly forms by electron microscopy. Capsomeres (L1-pentamers), T1-particles (12 L1-pentamers) and VLPs (T7 particles of 71 L1-pentamers) purified from infected insect cells expressing the wild-type HPV 16 L1 gene (L1wt) or mutated L1 (L1_2xCysM) were analyzed by electron microscopy after uranyl acetate negative staining (Thönes & Müller, 2007).

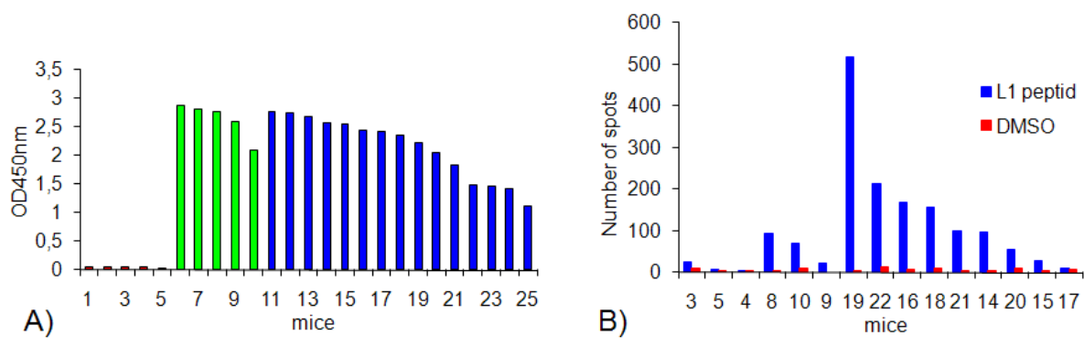


Fig. 11. Antibody (A) and cytotoxic T-lymphocytes (B) induction by L1-VPLs. Response elicited by control plant extracts (mice 1 - 5), control plant extract from *Nicotiana benthamiana* containing purified VLPs from insect cells (mice 6 - 10) and crude plant extracts from leaves expressing L1 (mice 11 - 25) in C57BL/6 mice. Collected sera from individual mice were tested by ELISA for induction of specific antibody (y-axis in OD 450 units). CTLs induction was measured by ELISPOT analysis of splenocytes recovered from scarified animals. The number of CTLs spots was recorded by an ELISPOT reader and expressed as a mean per 10^6 splenocytes

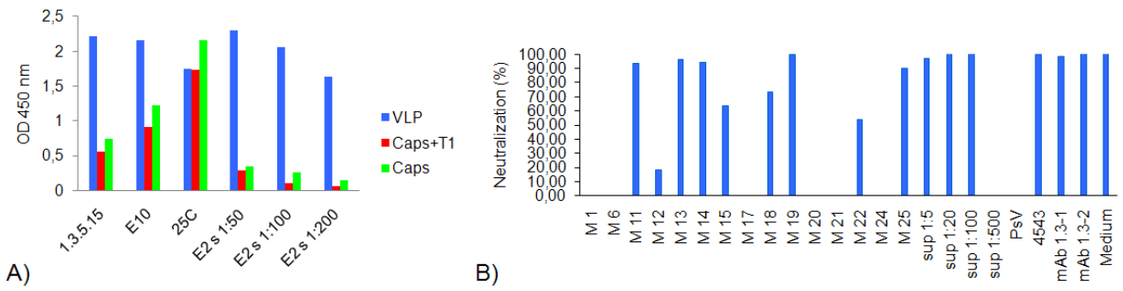


Fig. 12. (A) The specificity of mAb E2 toward various L1 assembly structures. VLPs, T1-particles (12 L1-pentamers) and capsomeres produced in insect cells were absorbed on the microtiter plates and analyzed by ELISA. Interactions of hybridoma E2 supernatant is compared to antibodies obtained after immunization with insect cell-derived VLPs (1.3.5.15, E10 and 25C). (B) Neutralization assay. Sera of mice and of E2 hybridoma supernatant were tested for neutralization of infection of 293T cells by HPV pseudovirions (Psv). Percent of neutralization obtained by incubation with mice sera diluted 1:50 and that of E2 hybridoma supernatant diluted 1:5, 1:20, 1:100, 1:500. Neutralization activity of mice sera was compared to a high titer rabbit polyclonal anti-L1 antiserum 4543 (100 %) and to the mAb's 1.3.1 and 1.3.2 as additional positive controls.

2.4.5 Plant based therapeutic and second-generation vaccines

Capsomeres can be used as a potential cost-saving substitute of VLPs, as L1-pentameric capsomeres are considered thermo-stable, the advantageous feature for the use in developing countries where cold chain administration and delivery of vaccine is difficult to maintain (Stanley et al., 2008). A promising step towards a capsomeres-based vaccine was described by Yuan et al. (2001) when dogs were completely protected against canine oral papillomavirus (COPV) infection by capsomeres vaccination. Capsomeres have been also reported to induce neutralizing antibodies and L1-specific cytotoxic T-lymphocytes (CTLs) upon oral, intranasal and subcutaneous immunization (Dell et al., 2006; Thönes & Müller, 2007; Schadlich et al., 2009). To increase immunogenicity, L1_2xCys mutant version of HPV16 L1 protein was fused with LTB as an adjuvant and expressed in chloroplasts (Waheed et al, 2011a, 2011b).

Plant expressed HPV antigens to be used as therapeutic vaccine to date are summarized in Table 3:

Antigen	Production system and yield	Efficacy data
HPV-16 E7	*N. benthamiana* tobacco leaves infected with PVX–E7 3–4 µg/g fresh leaves	40% of mice immunized with E7-containing crude leaf extract were protected from growth of cancer induced by E7-expressing C3 cells
HPV-16 E7	*N. benthamiana* tobacco leaves infected with PVX–E7; protein targeted to secretory pathway 15–20 µg/g fresh leaves	80% of mice immunized with E7-containing crude leaf extract were protected from growth of cancer induced by E7-expressing C3 cells
HPV-16 E7	*N. benthamiana* tobacco leaves infected with PVX–E7; protein targeted to secretory pathway	Mice vaccinated orally with freeze-dried E7-containing leaf extract mixed with feed produced high titer of anti-E7 antibodies
HPV-16 E7	*N. benthamiana* tobacco leaves infected with PVX–E7	Dendritic cells pulsed with E7-containing leaf extract were able to prime naive lymphocytes to induce E7-specific CTLs
LicKM–E7GGG	*N. benthamiana* tobacco leaves infected with LicKM–E7GGG, using a launch vector expression system 400 µg purified protein per gram of fresh leaves	Purified protein injected into mice induced IgG and CTL response and protected them against challenge with E7-expressing tumor cells in both prophylactic and therapeutic vaccination regimen
11-kDa Zera zein-derived peptide–E7 mut	*N. benthamiana* via agroinfiltration	Mice vaccinated with the protein were protected against tumor cells expressing E7
PVX CP–HPV-16 E7GGG	DNA vaccine	DNA vaccine was able to protect vaccinated mice from the growth of tumors induced by E7-expressing TC-1 cells
PVX CP–HPV-16 E7	Tobacco chloroplast	ND
SAP-KQ–E7GGG	a) DNA vaccine b) Expressed in N. benthamiana	a) DNA vaccine tested in therapeutic setting was able to block tumor growth in the 40% of challenged mice b) Not performed
HPV-16 L2–PVA CP–E7 epitope fused protein	Expressed by PVX in Nicotiana tabacum, N. benthamiana using Agrobacterium tumefaciens-mediated inoculation	ND
HPV-16 VLPs carrying L1 fused to a string of epitopes from E6 and E7	Tomato seedling cotyledons HPV-16 VLPs carrying L1 fused to string of epitopes from E6 and E7 using A. tumefaciens-mediated inoculation	Mice injected with chimeric VLPs were able to develop neutralizing antibodies and specific CTLs

Table 3. Plant-derived HPV antigens for the development of therapeutic vaccines. CP: Coat protein; CTL: Cytotoxic T lymphocyte; HPV: Human papillomavirus; ND: No data; PVA: Potato virus A; PVX: Potato virus X; SAP-KQ: Mutagenized type I ribosome inhibiting proteins from *Saponaria officinalis;* VLP: Virus-like particle. (Giorgi et al., 2010)

The important proof of the L2 plant-produced nonhuman papillomavirus vaccine efficiency was published in connection with the purified rTMV particles displaying cottontail rabbit papillomavirus (CRPV) or rabbit oral papillomavirus (ROPV) L2 protein peptides on their

surface (Palmer et al., 2006). This pseudovirion system was also used to express entire native *L1* gene of CRPV. Intramuscular injection with of CRPV L1-containing concentrated plant extract derived from transgenic tobacco protected rabbits against CRPV challenge (Kohl et al., 2006).

Since VLPs based vaccines are not effective in the therapy of diseases, an important goal is development of anti-HPV vaccines with either therapeutic or both prophylactic and therapeutic properties. Few studies were performed with viral oncoproteins expressed in plants. HPV-16 E7 was transiently expressed using a viral vector based on potato virus X (PVX) in the *Nicotiana benthamiana, Nicotiana rustica, Nicotiana tabacum, Chenopodium quinoa* and the tomato *Solanum lycopersicum L.* cv. Micro-Tom. The highest expression of HPV-16 E7 3–4 μg/g of fresh leaves was achieved in *Nicotiana benthamiana* and the expressed E7 induced specific humoral and cell-mediated immune responses in mice (Franconi et al., 2002, 2006). The efficiency of chimeric constructs when E7 is fused to other proteins and expressed in plants was also reported. The expression of HPV-16 E7 fused with the PVX CP in tobacco chloroplasts has been recently reported. The expression of the fusion protein in this system was higher than E7 alone (Morgenfeld et al., 2009). Mutated E7, E7GGG, which lacks the retinoblastoma binding site, and thus the native transformation potential, was fused to the *Clostridium thermocellum* b-1,3–1,4-glucanase (LicKM) as a carrier molecule for expression in plants. The expression of fusion protein in *Nicotiana benthamiana* yielded 400 μg of purified protein per gram of leaf (Musiychuk et al., 2007). Injection of the purified LicKM–E7GGG fusion protein into mice induced both E7-specific IgG and cytotoxic T-cell responses, and protected mice against challenge with E7-expressing tumor cells (Massa et al., 2007). The successful expression of chimeric HPV-16 L1 protein fused to a string of three E7 and one E6 epitopes in transgenic tomatoes demonstrates that a combination prophylactic / therapeutic HPV vaccine could be produced in plants (Paz de la Rosa et al., 2009; Monroy-García et. al, 2011). Unfortunately, while the produced VLPs stimulated both antibody and T-cell responses, yields were low in the range 0.05 – 0.1% of total soluble protein. Similarly, combined vaccine based on minor capsid protein L2 and an epitope of E7 oncoprotein was successfully expressed in plants, when an epitope of the L2 protein and an epitope of E7 oncoprotein were fused to the N'- and C'-end of PVA CP, respectively. The construct was cloned into a PVX-based vector and transiently expressed in plants using *Agrobacterium*-mediated inoculation (Čeřovská et al., 2008).

3. Vaccine formulation

3.1 Parenteral administration

3.1.1 Adjuvants

The goal of vaccination is to generate a strong immune response to the administered antigen. Papillomavirus VLPs themselves are good "inducers" of immune response and antigen determinants present on their surface are able to activate dendritic cells (DCs) for triggering T-cell activation (Bontkes et al., 2005; Yang et al., 2005). Nevertheless, for efficient clinical use additional adjuvants are needed not only to enhance the immune response, but also assuring achievement of appropriate type of protective immunity in each situation.

The aluminum (alum) salts or gel-based adjuvant formulations used e.g. in HPV Gardasil vaccine are currently approved in vaccines licensed for human use in the US. Nevertheless a

significant number of compounds tested for adjuvant effect are clearly more effective than alum, albeit usually accompanied with a higher toxicity as e.g. Freund's complete adjuvant. This is the main reason preventing their use as adjuvants in human vaccine formulations. As adjuvants were successfully tested low toxic mutants of the cholera toxin (CT) (Yamamoto et al., 1997) and *E.coli* heat-labile enterotoxin (LT) (Chong et al., 1998). The inactive B-subunits of these toxins proved to be a strong mucosal (oral, nasal, vaginal, etc.) adjuvants (e.g. Salmonella toxin B subunit is used in commercial Cervarix vaccine) for a wide variety of antigens in mice and other animal species so far tested, however their use in humans is limited (Chong et al., 1998). This is why there is a growing interest to develop new adjuvants eliciting high mucosal, humoral and cellular immune response accompanied by negligible or low toxicity.

Antigen delivery systems	**Immunopotentiators**
Insoluble aluminum compounds	MPL and synthetic derivates
Calcium phosphate	MDP and derivatives
Liposomes	Oligonucleotides (CpG, etc.)
Virosomes™	Double-stranded RNA (dsRNA)
ISCOMS®	Alternative pathogen-associated Molecular patterns (PAMPs) (*E. coli* heat Labile enterotoxin (LTB); flagellin)
Microparticles (e.g., PLG)	Saponins (Quils, QS-21)
Emulsions (e.g., MF59, Montanides)	Small-molecule immune potentiators (SMIPs) (e.g., resiquimod [R848])
Virus-like particles & viral vectors	Cytokines & chemokines

Table 4. Examples of adjuvant classes (O'Hagan & Rappuoli, 2004)

3.1.2 Adjuvant effect of plant extracts

Plant extracts are known to contain various compounds, which supposedly have immunostimulatory and immunosuppressive effects (Wagner & Proksh, 1985). Plant crude extracts and their components were tested for their adjuvant capacity. The extract from leaves of *Nicotiana bethamiana* co-administrated subcutaneously with HPV16E7 (Franconi et al., 2002) or HPV16L1 VLP´s (Maclean et. al, 2007; Šmídková et al., 2010) enhanced specific humoral and cellular immune response in tested mice. Freund's adjuvant added to the plant extract did not increased noticeably humoral response elicited by HPV16L1 VLP´s in subcutaneously immunized mice and results indicate that the addition of Freund's adjuvant to plant extract might be even deleterious (Maclean et. al, 2007). The study of Isfar et al. (2004) compares adjuvant effect of CT and of aqueous extract of *Solanum torvum* (STE). STE was shown to evoke an increase in IgA titer comparable to that of CT when co-administrated with ovalbumin intraperitoneally. No acute toxic effects were evident with the used dose range. Plant extract has been shown to induce DC maturation of dendritic cells. This effect was not caused by lipopolysaccharide (LPS) but rather by presence of heat-resistant products mimicking the effect of LPS in foliar extract (Di Bonito et al, 2009).

Probably the most studied plant compound with adjuvant effect is the saponin fractions isolated from *Quillaja saponaria* (Newman et al., 1992). The mechanism of saponin effect is complex and, apart from direct cellular stimulation, there is also evidence that saponins may

enhance oral immunization by protection of antigen from degradation by digestive enzymes and by increasing permeability of the intestine to macromolecules (Campbell, 1995).

All these findings are promising for development of needle-free administration route of immunization as an alternative to intramuscular vaccine application. For this purposes intranasal, intravaginal, transdermal, sublingual and intramuscular administration routes were tested for systemic immune responses against HPV16L1 using (Cho et al., 2010). The sublingual route provided the most effective mucosal secretory IgA (sIgA) and serum IgG responses, cholera toxin subunit B (CTB) showed the most promising adjuvant activity.

3.2 HPV L1 antigens as an edible vaccine?

The majority of currently licensed vaccines are administered parenterally, even though they have the disadvantages of patient reluctance to tolerate needle sticks and lack of mucosal immune induction (Velasquez et al 2010). Edible vaccine represents further approach to self-administrated nonparenteral vaccine that could solve the problem of high cost and need for appropriate storage of currently available preventive HPV vaccines.

Thönes & Müller (2007) investigated the oral immunogenicity of different assembly forms of HPV 16 L1: T7-VLPs, T1 particles and capsomeres produced from Baculovirus expression vector in insect cells and showed that all three assembly forms induce humoral and cellular immune responses after oral vaccination of mice. The anti-L1 antibodies were conformation-specific and showed neutralizing activity in a pseudovirion-based assay. They also investigated whether adjuvants have an effect on oral immunogenicity when co-administrated with different L1 forms. Besides saponins, which were significantly toxic if applied orally, co-administration of either CpG DNA or *Escherichia coli* heat-labile enterotoxin LT(R192G) had no apparent enhancing effect on the production of anti-L1 antibodies. Compared to capsomeres, VLPs induced stronger humoral immune responses while the CTL responses were induced at comparable levels.

To establish an edible HPV16 vaccine Sasagawa et al., (2005) constructed a recombinant HPV16 L1-expressing *Schizosaccharomyces pombe* yeast strain to be administrated as freeze-dried yeast powder orally as an edible vaccine, with or without the mucosal adjuvant heat-labile toxin LT (R192G), to mice. After the third immunization, none of the mice that received the edible HPV16 vaccine showed specific antibody responses, whereas all of the positive controls that were administered intranasally with 5 μg of HPV16-virus-like particles (VLP) had serum IgG, and genital IgA and IgG that reacted with HPV16-VLP in enzyme-linked immunosorbent assays (ELISAs).

HPV L1 antigens that proof to be highly immunogenic when administrated parenterally induce only mild or none response when administrated orally. In light of these experiments it seems unlikely that current design of L1 based HPV vaccines will reach the market as an edible replacement of existing vaccines. More research is needed to establish vaccine concentration and formulation to boost its effect. It is also obvious that the highly phrased concept of edible vaccine administrated as plants or fruits for direct consumption in the less developed countries is rather romantic dream than reality and have to be corrected. It is now clear that if there will be an edible vaccine, it will have complex formulation that will be strictly controlled.

A good nonparenteral alternative for vaccine delivery could be nasal immunization, which already proved to be effective in tests with animals. The obstacles imposed by the normal process of mucociliary clearance limiting residence time of applied antigens could be circumvent by presence of an inert *in situ* gelling polysaccharide (GelSite) extracted from Aloe vera for nasal delivery of NV VLP antigen (Hefferon, 2010). The nasal cavity is a promising site for vaccine delivery because it is easy to access, is highly vascularized, has a relatively large surface area, has low proteolytic activity, and is able to induce systemic immunity as well as both local and distal mucosal immunity via the Common Mucosal Immune System (CMIS)

4. Conclusions

The major reason for the vaccine production in plants is that the vaccine antigen production is safe and could be potentially cheap and both transient and transgenic productions are scalable. Biologically active proteins can be produced more easily in plants than in other eukaryotic systems; and that the use of food plants could eventually allow edible and/or oral vaccines to be produced cheaper. The recent reports indicate very high yields of human vaccine candidates to be obtained via plastid transformation or large scale transient expression what could enable to meet the expected requirement of antigen for oral route as is required parenterally for the same immune response. A recent review on human trials of plant-based oral vaccines summarizing human studies of oral transgenic plant derived vaccines against enterotoxigenic *E. coli* infection, norovirus and HBV adds weight to the growing body of evidence that plant-made oral vaccines to these viruses are not only feasible, but could effective (Rybicki, 2010). Nevertheless there is still long way to go from improvement of antigen yields, to formulation of the vaccine including auxiliary factors improving efficacy and stability, to translation of the proposed vaccines into clinical trials and, not least, governmental and/or regulatory body approvals.

5. Acknowledgements

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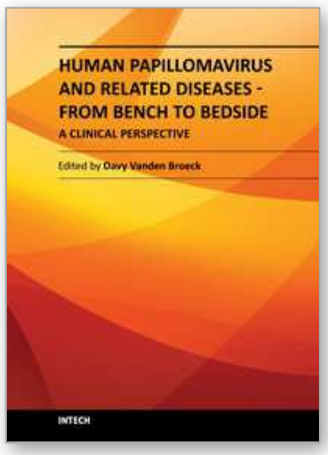
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Human Papillomavirus and Related Diseases - From Bench to Bedside - A Clinical Perspective

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Cervical cancer is the second most prevalent cancer among women worldwide, and infection with Human Papilloma Virus (HPV) has been identified as the causal agent for this condition. The natural history of cervical cancer is characterized by slow disease progression, rendering the condition, in essence, preventable and even treatable when diagnosed in early stages. Pap smear and the recently introduced prophylactic vaccines are the most prominent prevention options, but despite the availability of these primary and secondary screening tools, the global burden of disease is unfortunately still very high. This book will focus on the clinical aspects of HPV and related disease, highlighting the latest developments in this field.

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