EXPERT REVIEWS

Human papillomavirus vaccines in plants

Colomba Giorgi[†1],
Rosella Franconi[2] and
Edward P Rybicki[3,4]

[1]*Istituto Superiore di Sanità, Department of Infectious, Parasitic and Immunomediated Diseases, Viale Regina Elena 299, Rome 00161, Italy*
[2]*Italian National Agency for New Technologies, Energy and Sustainable Economic Development (ENEA), UTBIORAD, Laboratory of Biotechnology, CR Casaccia, Via Anguillarese 301, Rome, 00123, Italy*
[3]*Institute of Infectious Disease and Molecular Medicine, Faculty of Health Sciences, University of Cape Town, PB X3, Rondebosch 7701, South Africa*
[4]*Department of Molecular & Cell Biology, University of Cape Town, PB X3, Rondebosch 7701, South Africa*
[†]*Author for correspondence:*
Tel.: +39 064 990 3265
Fax: +39 064 990 2082
colomba.giorgi@iss.it

Human papillomaviruses are the etiological agents of cervical cancer, one of the two most prevalent cancers in women in developing countries. Currently available prophylactic vaccines are based on the L1 major capsid protein, which forms virus-like particles when expressed in yeast and insect cell lines. Despite their recognized efficacy, there are significant shortcomings: the vaccines are expensive, include only two oncogenic virus types, are delivered via intramuscular injection and require a cold chain. Plant expression systems may provide ways of overcoming some of these problems, in particular the expense. In this article, we report recent promising advances in the production of prophylactic and therapeutic vaccines against human papillomavirus by expression of the relevant antigens in plants, and discuss future prospects for the use of such vaccines.

The so-called 'high-risk' human papillomaviruses (HPVs) are responsible for causing cancer of the cervix in women, and are implicated in anogenital cancers and a significant percentage (25%) of head and neck tumors in men and women. Globally, HPVs can be considered the causative agents of 5% of all cancers. Cervical cancer is the second most prevalent cancer in women worldwide, and was the first cancer recognized by the WHO to be 100% attributable to an infectious agent [1,2]. In 2007, the American Cancer Society estimated an annual incidence of more than 500,000 new cervical cancer cases and approximately 300,000 deaths [3]. Prevention and early detection of cervical cancer relies on cytology screening programs, which, whenever available, have markedly reduced cervical cancer death rates. However, more than 80% of cervical cancer occurs in the developing world, where neither population screening nor optimal treatment is available. Furthermore, present treatments and, to a large extent, present screening strategies often do not acknowledge the viral etiology of this common cancer.

For early-stage cervical cancer, two radically different yet equally efficacious treatments can be offered to the patients: surgery and radiation therapy. Pelvic radiation in combination with chemotherapy represents the current standard therapy for the treatment of locally advanced disease [4]. However, despite technological advances, more than a third of patients will develop recurrence/metastatic disease, for which treatment results are poor.

The most efficient and cost-effective method for fighting human and animal infectious diseases over the years has been vaccination against the infectious agents. This can potentially prevent infection from occurring at all, or prevent spread of the agent in the infected host, through neutralization of infection mediated by antibodies as well as clearing infections via the cell-mediated immune response [5]. Traditional viral vaccines are based on live, killed or otherwise attenuated/modified pathogens or their components. Owing to the firm association of cervical cancer and HPV infection, the rapid development of a vaccine that could counteract the viral infection and consequently the associated cancer was expected. However, the fact that replication of HPV requires epithelial cell differentiation [6] has been a major hindrance in the *in vitro* production of virus, preventing conventional approaches for vaccine production.

With the development of DNA recombinant methodologies, traditional vaccines have now been frequently supplanted by recombinant subunit vaccines. This approach consists of the expression in a heterologous system of a gene encoding a protective antigen followed by purification of the resulting protein, which is then used as the vaccine [7].

Prophylactic vaccines against HPV infection

The history of prophylactic vaccines against HPV starts when the L1 viral protein, the principal component of the viral capsid, was shown to self-assemble to form structures resembling viral particles, the so-called virus-like particles (VLPs) [8]. After many years of research, two prophylactic vaccines are now available: these are the yeast-produced Gardasil® from Merck (NJ, USA), protecting against HPV types 16, 18, 6 and 11, and GlaxoSmithKline's Cervarix® (London, UK), made in insect cells via recombinant baculoviruses, and protecting against HPV-16 and -18. The two high-risk HPV types, HPV-16 and -18, are together responsible for approximately 70% of all cervical cancers [9]. Vaccines have shown excellent protective efficacy against the development of high-grade cancer intraepithelial neoplasia (CIN) 2/3 associated with HPV-16 and -18 [10,11]. Nevertheless, there are issues with both vaccines [12]: the VLP-based vaccines are expensive, making their use in developing countries problematic; they include only two oncogenic virus types; they are not efficacious against already established infections; they are delivered via intramuscular injection; and they require a cold chain. The challenges for second-generation vaccines will be the following. They should:

- Broaden coverage to elicit protection against not only HPV-16 and -18, but most of the other genital oncogenic HPV types as well;

- Induce long-term protection (for decades) at mucosal surfaces, while maintaining almost 100% efficacy of the current products;

- Be cheap, thermostable and, hopefully, deliverable via noninjectable methods;

- Provide therapeutic as well as prophylactic efficacy.

Moreover, if vaccination could also be extended to men and to older women, the eradication of HPV would be significantly accelerated [13].

There are a number of second-generation prophylactic HPV vaccines under investigation, made via bacterial and yeast fermentation, and via recombinant baculoviruses in insect cells. These include mixtures of more types of yeast-made VLPs from Merck that are already in trial, as well as more experimental vaccines in an earlier phase of development, such as capsomere-based products, chimeric L1 proteins designed to broaden the spectrum of protection, and vaccines including all or parts of the L2 minor capsid protein [14,15]. While DNA vaccines have also been investigated, their application seems to lie more in the province of therapy than prophylaxis, as they elicit only weak antibody responses.

Therapeutic vaccines against HPV-associated lesions

Therapeutic vaccines have a very different application to prophylactic vaccines: they are given to people already infected by the disease agent rather than healthy individuals, and so are designed to combat infection already established or disease progression, rather than preventing it. For this reason, they will generally seek to elicit cell-mediated immunity preferentially, as this is associated with clearance of infection rather than neutralization of the infectious agent. Therapeutic vaccines provide certain advantages over conventional therapeutic approaches: they are very specific, are generally well tolerated and usually have long-term effects. Moreover, immunotherapies could be combined with the conventional therapeutic approaches, leading to a more effective therapy against HPV-related cancer lesions.

A general immunotherapeutic approach against cancer consists of stimulating the immune system against target antigens present in the cancer cells. In the case of HPV-induced cancers, the viral target antigens are the E6 and E7 proteins, which are necessary to initiate and maintain the cell's transformed state [16], and which are accordingly constitutively expressed in tumors [17].

These proteins contribute to the events leading to cellular malignant transformation by interacting with their cellular target proteins with the ultimate effect of deregulation of cell cycle control, apoptosis control, terminal cell differentiation and antiviral defense [18,19]. These strategies are linked to evasion of the host immunosurveillance, so as to allow viral persistence, and facilitate the accumulation of chromosomal rearrangements [20]. The possibility of using viral antigens as therapeutic vaccines is attractive, because the induced immune response can be expected to be both potent and specific, given that the proteins are unrelated to the host repertoire. Several vaccine platforms specifically targeting E7 and/or E6 have been developed over the last decade [21,22], including peptide/protein-based therapies [23,24], viral vector-based therapies [25], DNA vaccines [26], dendritic cell (DC)-based immunotherapies [27,28] and chimeric VLPs [29,30].

Numerous challenges have to be addressed for the development of immune-based therapies for HPV-associated diseases. Among these are the development of more targeted adjuvants, strategies for better delivery and presentation of tumor antigens, and improvement of trafficking of effector T-cell populations at noninflamed mucosal sites and clinical translation [22]. Vaccines evaluated up until now in clinical trials have been shown to be moderately successful in eliciting cell-mediated immune responses to E6 and E7 in patients with a spectrum of HPV-associated diseases; however, the immune response was shown to not always correlate with clinical response [22,31]. Recently, the achievement of clinical responses in women with HPV-16-positive, grade 3 vulvar intraepithelial neoplasia (VIN3) by vaccination with a synthetic long-peptide vaccine against the HPV-16 oncoproteins E6 and E7 has been reported. Importantly, the complete responses were correlated with induction of HPV-16-specific immunity [24]. At present, however, there are no therapeutic vaccines against HPV licensed for use in humans. While this may be partially due to the preliminary nature of some of the investigations into their efficacy, it is also at least partially due to the uneconomic prospect for the exploitation of vaccines that will very probably be highly expensive if produced by conventional methods.

Vaccine antigen expression in plants

Parenterally administered vaccines such as the currently available HPV products are expensive to produce and costly to administer, which hampers their use especially in poor countries. A promising strategy for producing affordable vaccines involves

the expression of recombinant immunogens in plants. The principal justifications of this strategy are that antigen production in plants is safe and potentially very cheap, it can be very quick, and it is infinitely scalable [32–34]. Moreover, this strategy offers the possibility to express the vaccine antigens in food plants, or to formulate lightly processed antigen, potentially allowing oral vaccination; this strategy could help overcome present logistical and infrastructural problems of vaccine delivery and preservation, especially present in the poor regions where vaccines are needed most. Plants are a potential source of a very wide range of biopharmaceuticals [35], and a distinct advantage over bacterial fermenter-produced molecules is that protein products generally do not need downstream processing technology to ensure protein folding, particle assembly and stability. In consequence, a plant-based biopharmaceutical expression system makes oral dosing for prophylactic and/or therapeutic vaccination possible, either by simply feeding with edible plant tissues, or with only partially purified preparations, both of which would significantly reduce processing costs.

A number of prospective vaccine antigens from different origins have been successfully expressed in various plants: these include several animal virus proteins used in protection experiments (e.g., rabbit hemorrhagic fever virus and foot-and-mouth disease virus antigens), as well as human virus antigens such as hepatitis B surface antigen and – very recently – human norovirus capsid protein [33,36,37]. HPV antigens have been no exception: indeed, these were quite early targets of expression in plants, and both structural and nonstructural proteins have been successfully expressed, and in several cases used in model systems to prove efficacy. This article will cover the production and use of plant-derived prophylactic and therapeutic papillomavirus vaccine candidates, and will discuss future prospects for combined vaccines.

Production in plants of HPV capsid proteins for the development of prophylactic vaccines

Possibly the first published evidence that HPV L1 could be produced so as to assemble in transgenic plants came in the form of a US patent from Genomine Inc., Korea [101], filed in 2000: these authors claimed the production of L1 and L2 proteins of HPV-16 and -18 in transgenic tobacco (*Nicotiana tabacum*) and tomato (*Lycopersicon esculentum*), and assembly of (presumably L1-only) VLPs in both plants, and successful demonstration of immunogenicity in mice of both intraperitoneally injected and orally administered purified VLPs. No demonstration of L1+L2 VLP assembly was given, no description of the yields was given and the material was never published in another form.

While it did not involve production in plants, the next development of importance to second-generation and especially orally delivered HPV vaccines was the demonstration that HPV VLPs were orally immunogenic in mice [38], and that simple mucosal adjuvants significantly enhanced oral immunogenicity of HPV VLPs in mice [39], allowing a tenfold reduction in dose, from 100 to 10 µg or less. An important development from this has been the finding that insect cell-produced capsomers are also orally immunogenic [40].

The first literature reports of the making of HPV VLPs in plants, and the testing of their immunogenicity, were in 2003: three groups more or less simultaneously published accounts of the investigation of HPV L1 expression in transgenic plants. Varsani *et al.* expressed a full-length native *HPV-16 L1* gene in transgenic *N. tabacum* cv. Xanthi. They achieved a maximum of approximately 4 µg/kg wet weight (ww) of leaves of HPV-16 L1 protein as assayed by enzyme immunoassays: this protein assembled into recognizable VLPs (Figure 1), reacted with conformation-specific monoclonal antibodies (mAbs) and was immunogenic, albeit weakly, when injected into rabbits with Freund's incomplete adjuvant (FIA) [41]. The second group also reported the production of relatively low levels of protein, approximately 20 µg/kg, from a putatively plant codon-optimized *HPV-11 L1* protein gene in transgenic potato tubers: this assembled into recognizable VLPs, and was weakly immunogenic as an unconcentrated orally delivered vaccine in mice [42]. The third group took advantage of the previously reported fortuitous discovery that the use of human codon preferences for a *HPV-16 L1* gene yielded significantly better than either native or plant codon-optimized versions, to express HPV-16 L1 protein in transgenic tobacco and potato to levels of approximately 12 mg/kg in potato tubers and approximately 20 mg/kg in tobacco [43]. This was also
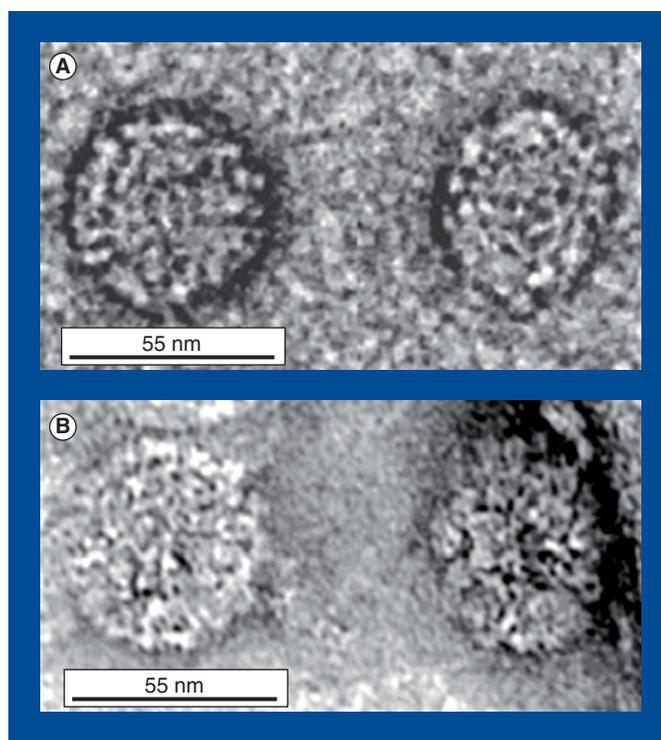

Figure 1. Comparison of plant- and insect-cell-produced human papillomavirus-16 L1 by electron microscopy. **(A)** Virus-like particles formed by human papillomavirus (HPV)-16 L1 protein expressed in *Sf*9 insect cells via recombinant baculovirus and purified by cesium chloride gradient centrifugation [42]. **(B)** Virus-like particles formed by HPV-16 L1 protein expressed in transgenic *Nicotiana tabacum* [38] and partially purified by cesium chloride gradient centrifugation. Both preparations were stained with phosphotungstate.

correctly assembled, and again was only weakly immunogenic when the potato product was used as an oral vaccine in mice. The tobacco-produced version, however, was highly immunogenic when injected as a cesium chloride gradient-purified product.

Another report of HPV-16 L1 production in transgenic tobacco [44], effectively, added nothing to the previous work: yields were very low (~0.05% of the total soluble protein [TSP]), and no new data on optimization or immunogenicity were provided. An attempt to increase the yield of HPV-16 L1 in *Nicotiana benthamiana* by use of a recombinant tobacco mosaic virus (rTMV)-derived vector system [45] was only partially successful: while yields of approximately 40 µg/kg could be obtained using the native gene, which represented a tenfold increase over transgenic production from the same gene, these were still too low for a viable production system.

Two important proofs of efficacy for plant-produced nonhuman papillomavirus vaccines were published just 3 months apart in 2006. The first described how rabbits could be completely or partially protected against cottontail rabbit papillomavirus (CRPV) infection by parenteral immunization with purified rTMV particles displaying CRPV or rabbit oral papillomavirus (ROPV) L2 protein-derived peptides, respectively, on their surfaces [46]. This was an important finding in that it demonstrated not only the utility of TMV as an antigen display vector, but also vaccine efficacy in an important preclinical disease model for papillomavirus infection, and that L2 protein could be an effective and at least partially cross-protecting vaccine. This study was also one of the first to use a novel technology for demonstration of antibody-based neutralization of papillomavirus infectivity: this is the transfected cell-produced pseudovirion system [47], which is now a necessary part of any papillomavirus vaccine investigation. The second proof of efficacy was of a more conventional CRPV L1-based vaccine: this described complete protection against CRPV elicited by intramuscular injection with FIA of CRPV L1-containing concentrated plant extracts derived either from transgenic *N. tabacum* cv. Xanthi, or from *N. benthamiana* infected with rTMV expressing the whole native *L1* gene [48]. This investigation was interesting for a number of reasons, all involving novel findings: first, the native gene expressed at levels up to 1 mg/kg in transgenic tobacco; second, use of a transient virus-based expression system did not increase yield, as the CRPV's gene was unstable in TMV; third, the expressed CRPV L1 protein did not detectably assemble into VLPs, while it did form conformationally relevant capsomeres; fourth, the vaccine protected against CRPV infection in the absence of any detectable neutralizing antibodies being elicited.

Other work from the same laboratory on another papillomavirus demonstrated that the native HPV-11 *L1* gene could be expressed at relatively high levels, and form capsomers and VLPs, in transgenic *N. tabacum* cv. Xanthi and *Arabidopsis thaliana* (2 and 12 mg/kg of L1 protein, respectively) and via rTMV expression in *N. benthamiana* (10 mg/kg) – but only via transgenic plants if the L1 open reading frame was truncated so as to remove the 3′-terminal 34 codons, encoding the nuclear localization signals (NLSs) [49] [Kohl T, Hitzeroth I, Stewart D *et al.*, Unpublished Data]. Moreover, the *N. tabacum* product was significantly proteolytically degraded compared with the *Arabidopsis*- or

N. benthamiana-produced L1, and antibodies elicited by injection of rabbits or guinea pigs with adjuvated concentrated plant extracts from the latter containing NLS-L1 cross-reacted only weakly with full-length insect cell-produced L1, and were not neutralizing in pseudovirion infection assays. Thus, while the work showed that another vaccine-relevant HPV L1 could be produced at reasonable levels in plants, it further showed that results obtained using HPV-16 could not necessarily be extrapolated to other papillomavirus L1 proteins – which may have important implications for making mixed VLP-type vaccines in plants. While the work reported above demonstrated important proofs of concept and, indeed, of vaccine efficacy, none of the reported yields of total L1 protein were above 20 mg/kg ww: this corresponds approximately with a value of 0.5% of the TSP, which is half of the often-quoted lower limit for commercially viable production of antibodies [50] and does not take into account the far lower yield of assembled VLPs. Accordingly, the Rybicki group systematically investigated differently codon-optimized versions of the HPV-16 *L1* gene, and differential intracellular targeting, by transient production via infiltration of *N. benthamiana* plants with recombinant *Agrobacterium tumefaciens*, in an effort to determine optimum conditions for production [51]. Of the three gene versions tried, native, plant and human codon usage-optimized – with tomato spotted wilt virus nonstructural small silencing suppressor protein coexpression – the human version (hL1) was by far the best; chloroplast targeting of L1 was also consistently the best option (>500 mg/kg; 17% of the TSP), although cytoplasmic expression of hL1 also gave acceptable yields (380 mg/kg; 15% of the TSP). Characterization of the products by electron microscopy and mAb binding showed appreciable accumulation of appropriately formed VLPs, and that most of the total L1 bound to an assembly-dependent. conformation-specific mAb. Moreover, parenteral immunization of mice with centrifugally concentrated plant extracts elicited as high-titer antisera (1:40,960) as could be achieved using insect-cell-produced VLPs, which had neutralizing antibody titers as assessed by inhibition of pseudovirion infectivity (1:1600–1:6400) that were in the same range as those reported for human trial subjects injected with commercial vaccines [Schiller J, Pers. Comm.]. Moreover, the test vaccine was stable after lyophilization, and neither the serum titers nor their neutralizing titers were improved by use of FIA. This work represented the best evidence to date that plant-based HPV L1-based vaccine production at exploitable levels was a feasible prospect, and that a candidate HPV vaccine could be successfully reconstituted after lyophilization.

An important sequel to this investigation was the proof that transgenic *N. tabacum* cv. Petit Havana plants duplicated the transient expression pattern, with plants expressing chloroplast-targeted L1 accumulating up to 650 mg/kg (11% of the TSP) of L1 – but that this happened only in the first (T1) generation [51]; from T2 onward, expression had apparently been silenced in all lines [Maclean J, Rybicki EP, Koekemoer M, Unpublished Data]. While this was disturbing, the fact that transient expression was so successful was encouraging, as this is also a feasible and increasingly exploited means of routine production of 'pharmed' proteins [33,37]. Further work in this regard

showed that a geminivirus-derived expression vector, introduced to *N. benthamiana* or potentially, a wider range of dicotyledonous plants via agroinfiltration, is also routinely able to enhance HPV-16 *hL1* gene production levels in the cytoplasm by 50% or more [52].

While transgenic production of L1 via nuclear transformation has been problematic in terms of achieving and maintaining yield, transplantomic expression has apparently been far more successful. For example, Lenzi *et al.* [53] showed that either native or a chloroplast-optimized HPV-16 *L1* gene could be produced at levels up to 1.5% of the TSP, and that protein both folded properly and formed capsomeres and VLPs. However, in order to achieve this result they found necessary to translationally fuse the N-terminus of the L1 protein with the first 14 amino acids of the N-terminal domain of the ATPase β-subunit or the Rubisco large subunit, and to remove the 22-amino acid C-terminal NLS. Another group achieved contrasting results; they found that no modification of the HPV-16 *L1* gene was necessary to achieve what is, presently, the highest plant-produced L1 yield ever recorded. Fernandez-San Millan and colleagues used a native HPV-16-derived *L1* gene transformed into tobacco chloroplasts to achieve yields of 3 g/kg ww (24% of the TSP) of L1 protein [54]. This assembled into VLPs, bound conformation-specific mAbs, and elicited neutralizing antibodies when mice were injected intraperitoneally with concentrated plant extracts together with Freund's or aluminium hydroxide adjuvants.

The expression of minor capsid HPV protein L2 in plants have been paid very little attention; it was reported in passing in the 2002 Korean patent [101]. Also reported was the production of a TMV-displayed rabbit papillomavirus L2 peptide [46]. With the increasing attention being paid to L2 protein as an important factor in broadening the otherwise highly type-specific protection elicited so efficiently by L1 assemblies, this may soon change. The HPV-16 L2 protein has been successfully expressed via agroinfiltration in *N. benthamiana*, as well as in transgenic *N. tabacum*: it was necessary to use a human codon-optimized gene rather than plant-optimized or the native version, as for HPV-16 *L1*, but the protein accumulated equally well via transient expression in chloroplasts, cytoplasm and endoplasmic reticulum, to a level of approximately 30 mg/kg [55] [Pereira R, Hitzeroth I, Rybicki EP, Unpublished Data]. Given that L2 protein coassembles with L1 at a molecular ratio of between 1 and 6:30 (i.e., a maximum of one L2 molecule per L1 capsomer [55]) and L1 yields are approximately 10–20-times higher, it is entirely feasible to suppose that coexpression via agroinfiltration of the two proteins could result in efficient assembly of L1+L2 VLPs.

The recent demonstration that concatenated multitype L2 fusion polypeptides, derived from known cross-protective epitopes of several divergent HPVs [56], elicited highly cross-neutralizing antisera, builds on other evidence that L1:L2 chimeric assembled molecules elicit more broadly cross-reactive antibodies than L1-only or even L1+L2 VLPs [42,57], and should spur much further research on chimeric L1:L2 protein expression. The former paper reports the investigation of insect cell-produced HPV-16 L1 molecules with a 13-residue HPV-16 L2-derived peptide (LVEETSFIDAGAP; L2 108–120) as second-generation HPV vaccines: production of these and other L1:L2 chimeras in plants is currently being investigated, with significant preliminary success [Whitehead M, Hitzeroth I, Rybicki EP, Unpublished Data]. A summary of the plant-expressed HPV antigens for the development of a prophylactic vaccine is reported in Table 1.

Production of HPV oncoproteins in plants for development of therapeutic vaccines

Plant-made therapeutic vaccines against challenging chronic diseases such as cancer have received little research attention. A notable exception, however, was the human Phase I clinical trial of plant-produced idiotype vaccines from Large Scale Biology Corp. (CA, USA) for the treatment of non-Hodgkin's lymphoma [58], demonstrating the safety and to some extent the efficacy of the individually tailored vaccines. The concept has been taken over by Icon Genetics and further trials are planned [36].

An ideal vaccine against HPV-associated cancer has to overcome the problem caused by the fact that the virus creates an immunosuppressive environment, leading to ineffective immune responses. A therapeutic cancer vaccine should trigger effector T-cell trafficking, overcome immunosuppression and generate acute inflammation at the tumor site [59]. Such requirements could be fulfilled by linking the tumor-specific antigen (TSA) to molecules able to increase its immune 'visibility'. Thus, one of the challenges for the development of HPV-specific therapeutic vaccines is the production and the delivery of the oncoproteins E6 and/or E7 in a suitable form to be recognized as TSAs in virus-associated neoplasia.

Few studies have been performed in this field with viral oncoproteins expressed in plants. In the first report, from 2002, HPV-16 E7 was transiently expressed in tobacco plant using a 'first-generation' viral vector based on potato virus X (PVX) [60]. Because foreign proteins are produced to very different levels in different PVX-susceptible plants [61], Franconi and colleagues examined E7 expression in the well-known PVX hosts *N. benthamiana*, *Nicotiana rustica*, *N. tabacum*, *Chenopodium quinoa* and the miniature *Lycopersicon esculentum* cultivar Micro-Tom. The E7 protein was expressed at highest levels in *N. benthamiana*, at 3–4 µg/g in fresh leaves [60].

In most of the plant expression systems the E7 of approximately 17 kDa appeared in high-molecular-mass forms that were resistant to sodium dodecyl sulfate treatment and boiling, suggesting its close interaction with plant components. A similar pattern was seen for E7 also in reconstituted samples containing uninfected *N. benthamiana* extracts and *Escherichia coli* His–E7 purified protein: in these samples, the formation of the high-molecular-mass forms was prevented only by an extensive heat treatment (100°C for 10–15 min) of the extract prior to the addition of purified His–E7, suggesting the involvement of heat-stable plant components.

The E7-containing crude foliar extract from *N. benthamiana* leaves infected with E7–PVX was used as vaccine in a mouse model [60]. Animals were immunized several times with a vaccine dose containing 0.5 µg of E7; control mice were immunized with 0.5 µg of *E. coli*-made recombinant His–E7 plus the adjuvant QuilA [62]. E7-specific humoral and cell-mediated immune responses were induced in the mice. The antitumor activity of the vaccine was

Table 1. Plant-derived human papillomavirus antigens for the development of prophylactic vaccines.

Antigen	Production system and yield	Efficacy data	Ref.
HPV-16 L1	*Agrobacterium*-transformed *Nicotiana tabacum* cv. Xanthi plants Assembled in VLPs 4 µg/kg ww	Weakly immunogenic in rabbit	[41]
HPV-11 L1	Transgenic potato tubers Assembled in VLPs 20 µg/kg	Weakly immunogenic in orally vaccinated mice	[42]
HPV-16 L1	Transgenic potato tubers Assembled in VLPs 12 mg/kg	Weakly immunogenic in orally vaccinated mice	[43]
HPV-16 L1	Transgenic tobacco plants Assembled in VLPs 20 mg/kg ww	Highly immunogenic in mice injected with purified product	[43]
HPV-16 L1	Protein expressed in *Nicotiana benthamiana* by TMV-derived vector 40 µg/kg wet leaves	ND	[45]
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	[48]
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	[51]
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	[53]
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	[56]

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.

evaluated by challenging vaccinated mice with C3 cells, an embryonic mouse cell line expressing the HPV-16 E7 oncoprotein [63]. Both vaccine preparations inhibited the tumor growth in 40% of vaccinated mice, whereas the control mice and mice vaccinated with foliar extract from plants infected with wild type PVX were all tumor-affected 7 days after the challenge. The antitumor activity of this vaccine preparation was improved by increasing the amount of E7 in the foliar crude extract. If PVX-expressed E7 was fused to a plant-derived secretory pathway signal sequence [64], the E7 protein expression level was enhanced fivefold. A total of 80% of mice immunized with this extract (2.5 µg of E7 per dose) were tumor-free 50 days after challenge with C3 cells; the protection was associated with a strong Th1 cell response. It was interesting that 100% of animals could be protected using a vaccine containing 10 µg – four-times the plant-derived dose – of *E. coli* His–E7 with QuilA adjuvant. These results strongly suggested an adjuvant-like activity of the foliar extract.

The E7 protein expressed in this system was also stable for at least 18 months when stored in lyophilized *N. benthamiana* leaves at room temperature. Freeze-dried E7-containing *N. benthamiana* leaf extract mixed with feed was used to vaccinate mice in an oral vaccination regimen [Franconi R *et al.*, Unpublished Data]. The extract

was not toxic and a high titer of anti-E7 antibodies was induced in the animals, suggesting the possibility of an oral immunization approach. This is being further followed-up.

The adjuvant-like activity of the plant extract and the possible use of an E7-containing plant extract in the immunotherapy of HPV-related lesions were investigated using human DCs, antigen-presenting cells that are major players in the induction of immune responses in the target tissues [65]. The extract was not toxic for the cells, did not influence E7 uptake into the cells, did not affect DC differentiation, but did induce DC maturation. This effect was not caused by lipopolysaccharide (LPS) but rather to the presence in the foliar extract of heat-resistant products mimicking the effect of LPS. Importantly, the E7-containing extract was able to prime naive lymphocytes to produce an E7-specific T-cell response. While this study gives some valuable insights about the immunomodulatory activity of the *N. benthamiana* plant extract in a preclinical model, its possible use and application in humans requires the characterization of the compounds present in the foliar extract – in terms of protein aggregates, protein folding differences, protein post-translational modifications, lectins, heat-shock proteins and lipids – that could contribute to its activity.

The effectiveness of chimeric constructs of E7 fused to other proteins and expressed in plants has also been reported. A mutant of E7, E7GGG, lacking the retinoblastoma protein-binding site and, thus, the native transformation potential [66], was fused to the *Clostridium thermocellum* β-1,3–1,4-glucanase (LicKM) as a carrier molecule for expression in plants. The fusion protein was expressed in *N. benthamiana* using a 'second-generation' viral vector ('launch vector') [67]. In this way, a yield of 400 µg purified protein per gram of leaf was obtained. Injection of the purified LicKM–E7GGG fusion protein into mice induced both E7-specific IgG and cytotoxic T-cell responses, and protected vaccinated mice against challenge with E7-expressing tumor cells [68]. Furthermore, therapeutic vaccination of a large number of animals (50 per treatment) with this antigen reproducibly prevented tumor growth, even in the presence of fully established tumors [69]. This antigen is being expressed in tobacco hairy root cultures, exploiting a virus-based approach [70,71]. The results of therapeutic vaccination indicate that LicKM–E7GGG fusion protein, purified from plants or from *in vitro* hairy root cultures could represent a powerful therapeutic formulation, opening the way to the possibility of a Phase I clinical trial in the near future. We note that the latter expression system is fully contained and more akin to recognized good manufacturing practice (GMP) protocols already accepted by regulatory agencies.

The plant yield- and immune-enhancing properties of the 11-kDa Zera® zein-derived peptide (Era Biotech, Barcelona, Spain), which promotes aggregation and sequestration of coupled proteins into in large (>1 µm) endoplasmic reticulum membrane-enveloped protein bodies in a variety of expression systems [72], have been tested in conjunction with a shuffled, inactive form of the HPV-16 E7 protein [73]. Preliminary results indicate that immunization of mice with purified Zera-conjugated shuffled E7, produced at high levels in *N. benthamiana* via agro-infiltration, protected them against challenge with tumor cells expressing E7, and caused regression of established tumors, at least as well as a DNA vaccine expressing shuffled E7 alone [Whitehead M *et al.* Unpublished Data].

Plant virus coat proteins (CPs) are often able to self-assemble into highly immunogenic structures, resembling pathogens. The PVX CP in particular has been shown to trigger antigen-specific CD4+ T-cell responses [74,75]. Taking advantage of this capacity, the PVX *CP* gene was translationally fused to E7GGG, and tested as a DNA vaccine [76]. Vaccination of mice with this DNA construct induced a significantly increased cell-mediated immune response, reduced growth of tumors induced by TC-1 cells [77] and enhanced mouse survival in therapeutic experiments compared with vaccination with the *E7GGG* gene alone. This novel design may represent an innovative way to enhance immunogenicity of the encoded product, reducing concerns about inappropriate immune responses or autoimmunity in therapeutic vaccination regimens designed to treat cervical cancer.

The expression in tobacco chloroplasts of HPV-16 E7 fused with the PVX CP has been recently reported [78]. The expression of the fusion protein in this system was higher than that of E7 alone, even though the transcript accumulation was lower. This suggests that the PVX CP could stabilize E7 in the chloroplast stroma, opening the way for its possible use as a therapeutic vaccine.

Besides plant virus CPs, other plant-derived sequences might also offer safe means of activating immunity against HPV. Ribosome-inhibiting proteins (RIPs), plant enzymes that depurinate rRNA inhibiting protein synthesis, could be just such carriers. The toxicity of RIPs has previously been exploited in medicine in order to prepare conjugates with antibodies that act as immunotoxins. However, the biological activity of RIPs is very wide, and much of this is independent of the inhibition of protein synthesis [79]. Features such as their ability to induce production of cytokines causing inflammation, to promote immune responses, their antiviral activity, their antigenicity, their apoptotic and necrotic potential, could make RIPs new, useful tools in tumor therapy. A mutagenized, single-chain type-I RIP, derived from *Saponaria officinalis* (SAP-KQ) and used as a carrier for HPV-16-derived E7GGG protein in a DNA vaccine, interfered with tumor growth by triggering E7-specific antibodies and cytotoxic T lymphocytes (CTLs) and delayed-type hypersensitivity [102]. These data open the way for the use of plant-derived carriers to improve antigen immunogenicity in the development of anticancer vaccines.

A summary of the plant-expressed antigens for the development of a therapeutic vaccine is reported in Table 2.

Combined prophylactic & therapeutic vaccines: an attractive possibility

Since the benefits of the actual prophylactic vaccination will only be visible after decades and since present preventive vaccines have no therapeutic effect, an important goal could be the development of anti-HPV vaccines with both prophylactic and therapeutic properties. These vaccines would probably have to contain epitopes from the viral CPs (L1 and/or L2) and from the oncoproteins (E6 and/or E7).

Cerovská and collaborators have recently proposed plant-based production of HPV antigens for the development of prophylactic and therapeutic vaccines [80]. They succeeded in expressing a recombinant potato virus A (PVA) CP carrying two different HPV-16 epitopes: an epitope of the minor CP L2 and an epitope of E7 oncoprotein were fused to the N-terminus and to the C-terminus of the PVA CP, respectively. The construct was cloned into a PVX-based vector and transiently expressed in plants using *A. tumefaciens*-mediated inoculation. Synergistic infection of host plants with PVX carrying the construct and potato virus Y increased the expression of L2-ACP-E7 in *N. tabacum* and in transgenic *N. benthamiana*.

In another study, Paz de la Rosa and collaborators reported the construction of a recombinant HPV-16 L1 protein fused to a string of three E7 and one E6 epitopes known to mediate CTL activity, and its expression in transgenic tomato [81]. This protein assembles into VLPs, even though its expression level is not high (0.05–0.1% of the TSP). VLP intraperitoneal immunization of mice elicited both neutralizing antibodies and CTL activity against the E6 and E7 epitopes. This is a good preliminary indication for the development of a combined prophylactic and therapeutic vaccine based on this construct (Table 2).

Table 2. Plant-derived human papillomavirus antigens for the development of therapeutic vaccines.

Antigen	Production system and yield	Efficacy data	Ref.
HPV-16 E7	*Nicotiana 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	[60]
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	[64]
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	[Franconi R *et al.*, Unpublished Data]
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	[65]
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	[68,69]
11-kDa Zera zein-derived peptide–E7 mut	*N. benthamiana* via agroinfiltration	Mice vaccinated with the protein were protected against tumor cells expressing E7	[Whithead *et al.*, Unpublished Data]
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	[76]
PVX CP–HPV-16 E7	Tobacco chloroplast	ND	[78]
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	[102] [Franconi *et al.*, Unpublished Data]
HPV-16 L2–PVA CP–E7 epitope fused protein	Expressed by PVX in *Nicotiana tabacum*, *N. benthamiana* using *Agrobacterium tumefaciens*-mediated inoculation	ND	[80]
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	[81]

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.

Conclusion

There is now abundant evidence that HPV and other papillomavirus L1 VLPs can be expressed in plants by several routes and at high yields, and that these are 'biosimilars' in terms of their morphology, immunogenicity and even efficacy in animal models, compared with the commercial yeast and insect cell-produced VLP-based prophylactic vaccines. There is evidence that plant-made preparations can be lyophilized, without losing their immunogenicity with or without adjuvant. Evidence that insect cell-produced VLPs and even capsomers are orally immunogenic lends weight to the use of the plant-made product in similar ways, probably at far lower expense, given the rapidly improving regulatory landscape for plant-made pharmaceuticals.

These considerations can also be applied to the production of L2 protein in plant, alone or combinations with L1 in chimeric proteins that are viable candidate second-generation vaccines.

The production in plants of a variety of candidate therapeutic and even combined prophylactic/therapeutic has also been amply investigated, with significant proofs of efficacy in accepted animal model systems. The case for development and human trial of these vaccines is possibly stronger than for prophylactic L1/L2 VLP or chimeric vaccines, given that the major incidence of HPV infection and related diseases is in developing countries, which requires the means of production to be as cheap as possible. In the case of HPV therapeutic vaccination, since no 'reference' vaccines are available at the moment and due to the complex

responses that a therapeutic vaccine should evoke, plants can be considered not only as potential biofactories but also as a source of immunomodulating molecules, which can create the proinflammatory environment necessary for the clearance of persistent HPV infection and related lesions. In this possible application, the use of plant-derived molecules should not provoke the safety concerns for clinical use that exist for some animal-derived immune response modifiers currently being tested in experimental studies.

Finally, it is evident that the breadth and depth of the investigation of the feasibility of production in plants of both prophylactic and/or therapeutic vaccines against HPV infection and HPV-caused cancer lesions, and the immunogenicity and efficacy of these vaccines, represents an excellent case study for the vaccine industry. Effective products have been made; immunogenicity and efficacy has been demonstrated; it requires only human trials to cement the need for this means of production to provide cheap vaccines.

Expert commentary

The prophylactic HPV vaccines now available have been shown to be successful in reducing the incidence of precancerous cervical lesions caused by HPV-16 and -18, but a number of critical problems remain: for example, how the vaccines affect the prevalence of other viral types, considering the possible selective pressure on other HPV strains if the vaccine strains are effectively suppressed; the absence of therapeutic efficacy; and, not least, the high cost of manufacture of present vaccines. The research community is very active in developing new-generation vaccines to answer these questions, and importantly, is also developing new biotechnology strategies to cope with the challenge of making low cost innovations for developing countries. The development of plant molecular farming in the last few years provides exciting new ways for the production of vaccines. Many recombinant proteins, also of biopharmaceutical interest, have been produced in different plant species, and many are approaching commercial approval.

The major reasons for vaccine production in plants are that vaccine antigen production is safe and potentially very cheap, and is infinitely scalable; that biologically active proteins can be produced more easily in plants than in other eukaryotic systems; and that the use of food plants could allow edible and/or oral vaccines to be locally and more cheaply produced.

These characteristics neatly fit the perceived need for innovative vaccines to protect against HPV infection and related diseases. Unfortunately, the path to product contains many obstacles, such as the improvement of antigen yields, the translation of the proposed vaccines into clinical trials and, not least, governmental and/or regulatory body approvals. However, we are convinced that plant-produced HPV vaccines will shortly take their place in the pharmacological arsenal.

Five-year view

Despite nearly 20 years of development of plant recombinant protein expression technology, there are only two plant-produced vaccine-related products that have passed all production and regulatory hurdles: these are a mAb used in the production of a recombinant hepatitis B virus vaccine, and a Newcastle disease virus vaccine for poultry. Thus, the idealistic vision of a plant-provisioned arsenal of vaccines and therapeutics for poor people may still be far from realization, due to problems linked to production and processing of the products, and to industry and public acceptance. However, the recent decisions of a big tobacco company to invest in the production of the seasonal and H5N1 influenza vaccines, and the acquisition by Pfizer (NY, USA) of the company making a niche mitochondrial-defect therapeutic, are very encouraging developments.

It is likely that the plant-derived vaccines that will be developed in the near future will not be copies of the existing ones such as that against hepatitis B virus infection that are already cheap, already licensed as generics and already included free in the Expanded Program for Immunization offerings of many countries, but will be generics or biosimilars directed against disease agents such as HPV and rotavirus, given the currently very high price of proprietary vaccines.

Key issues

- The second generation of human papillomavirus (HPV) prophylactic vaccines will have to be inexpensive as well as immunogenic, safe, stable and easy to administer.
- Therapeutic vaccines against HPV-associated lesions have to be able to induce specific, cell-mediated immune response against E6 and E7 viral oncoproteins.
- Plant molecular farming provides exciting new methods for the production of vaccines.
- Antigens expressed in food plants could allow the production of edible or at least oral vaccines.
- HPV antigens have been successfully expressed in different plant-based systems.
- HPV antigens present in plant extracts or purified from plants are immunogenic in animal models.
- Plant-produced HPV vaccines have been shown to be effective in preventing or limiting disease in model systems.

References

1   *IARC Monographs on the Evaluation of Carcinogen Risks to Humans: Human Papillomaviruses.* World Health Organization International Agency for Research on Cancer, Lyon, France, 64 (1995).

2   Bosch FX, Lorincz A, Munoz N, Mejier CJ, Shah KV. The causal relation between human papillomavirus and cervical cancer. *J. Clin. Pathol.* 55, 244–265 (2002).

3   Garcia M, Jemal A, Ward EM *et al. Global Cancer Facts and Figures 2007.* American Cancer Society, Atlanta, GA, USA (2007).

4   Thomas GM. Improved treatment for cervical cancer-concurrent chemotherapy and radiotherapy. *N. Eng. J. Med.* 340, 1198–1220 (1999).

5   Reading SA, Dimmock NJ. Neutralization of animal virus infectivity by antibody. *Arch. Virol.* 152, 1047–1059 (2007).

6   Zur Hausen H. Papillomavirus infections, a major cause of human cancers. *Biochem. Biophys. Acta.* 1288, F55–F58 (1996).

7   Plotkin SA. Vaccines: past, present and future. *Nat. Med.* 11(4), S5–S10 (2005).

8   Kirnbauer R, Booyt F, Cheng N, Lowy DR, Schiller JT. Papillomavirus L1 major capsid protein self-assembles into virus-like particles that are highly immunogenic. *Proc. Natl Acad. Sci. USA* 89, 12180–12184 (1992).

9   Bosch FX, Burchell AN, Schiffman M *et al.* Epidemiology and natural history of human papillomavirus infections and type-specific implications in cervical neoplasia. *Vaccine* 26(Suppl. 10), K1–K16 (2008).

10 Paavonen J, Jenkins D, Bosch FX *et al.* Efficacy of a prophylactic adjuvated bivalent L1 virus-like-particle vaccine against infection with human papillomavirus types 16 and 18 in young women: an interim analysis of a Phase III double-blind, randomised controlled trial. *Lancet* 369(9580), 2161–2170 (2007).

11 Garland SM, Hernandez-Avila M, Wheeler CM *et al.* Quadrivalent vaccine against human papillomavirus to prevent anogenital diseases. *N. Engl. J. Med.* 356(19), 1928–1924 (2007).

12 Haug CJ. Human papillomavirus vaccination – reasons for caution. *N. Engl. J. Med.* 359(8), 861–862 (2008).

13 Michels KB, zur Hausen H. HPV vaccine for all. *Lancet* 374(9686), 268–270 (2009).

14 Schiller JT, Nardelli-Haefliger D. Chapter 17, second generation HPV vaccines to prevent cervical cancer. *Vaccine* 24(Suppl. 3), 147–153 (2006).

15 Palmer KE, Jenson AB, Kouokam JC, Lasnik AB, Ghim SJ. Recombinant vaccines for the prevention of human papillomavirus infection and cervical cancer. *Exp. Mol. Pathol.* 86(3), 224–233 (2009).

16 Hudsun JB, Bedell MA, McCance DJ, Laiminis LA. Immortalization and altered differentiation of human keratinocytes *in vitro* by the E6 and E7 open reading frames of HPV18. *J. Virol.* 64, 519–526 (1999).

17 Zur Hausen H. Papillomaviruses causing cancer: evasion host-cell control in early events in carcinogenesis. *J. Natl. Cancer Inst.* 92(9), 690–698 (2000).

18 Fehermann F, Laimins LA. Human papillomaviruses: targeting differentiating epithelial cells for malignant transformation. *Oncogene* 22(33), 5201–5207 (2003).

19 Munger K, Baldwin A, Edwards KM *et al.* Mechanisms of human papillomavirus-induced oncogenesis. *J. Virol.* 78(21), 11451–11460 (2004).

20 Ghittoni R, Accardi R, Hasan U, Gheit T, Sylla B, Tommasino M. The biological properties of E6 and E7 oncoproteins from human papillomaviruses. *Virus Genes* 40(1), 1–13 (2010).

21 Brinkman JA, Hughes SH, Stone P *et al.* Therapeutic vaccination for HPV induced cancers. *Dis. Markers* 23, 337–352 (2007).

22 Trimble CL, Frazer IH. Development of therapeutic HPV vaccines. *Lancet Oncol.* 10, 975–980 (2009).

23 Bolhassani A, Mohit E, Rafati S. Different spectra of therapeutic vaccine development against HPV infections. *Hum. Vaccin.* 5(10), 671–689 (2009).

24 Kenter GG, Welters MJ, Valentijn AR *et al.* Vaccination against HPV-16 oncoproteins for vulvar intraepithelial neoplasia. *N. Engl. J. Med.* 361(19), 1838–1847 (2009).

25 Zhou L, Zhu T, Ye X *et al.* Long term protection against HPV E7-positive tumor by a single vaccination of AAV vectors encoting a fusion protein of inactivated E7 of HPV16/18 and heat shock protein 70. *Hum. Gene Ther.* 21(1), 109–119 (2010).

26 Wu TC. Therapeutic human papillomavirus DNA vaccination strategies to control cervical cancer. *Eur. J. Immunol.* 37, 310–314 (2007)

27 Bellone S, Pecorelli S, Cannon MJ, Santin AD. Advances in dendritic cell-based therapeutic vaccines for cervical cancer. *Expert Rev. Anticancer Ther.* 7(10), 1473–1486 (2007).

28 Monie A, Tsen SW, Hung CF, Wu TC. Therapeutic HPV DNA vaccines. *Expert Rev. Vaccines* 8(9), 1221–1235 (2009).

29 Kaufman AM, Nieland JD, Jochmus I *et al.* Vaccination trial with HPV16 L1E7 chimeric virus-like particles in women suffering from high grade cervical intraepithelial neoplasia (CIN 2/3). *Int. J. Cancer* 121(12), 2794–2800 (2007).

30 Di Bonito P, Grasso F, Mochi S *et al.* Anti-tumor CD8+ T cell immunity elicited by HIV-1-based virus-like particles incorporating HPV-16 E7 protein. *Virology* 395(1), 45–55 (2009).

31 Hung CF, Monie A, Tsen SW, Wu TC. Therapeutic human papillomavirus vaccines: current clinical trials and future directions. *Expert Opin. Biol. Ther.* 8(4), 421–439 (2008).

32 Gomez Lim MA. Transgenic plants in therapeutically valuable protein production. *Transgenic Plant J.* 1, 256–266 (2007).

33 Rybicki EP. Plant-produced vaccines: promise and reality. *Drug Discov. Today* 14(1/2), 16–24 (2009).

34 Yusibov V, Rabindran S. Recent progress in the development of plant-derived vaccines. *Expert Rev. Vaccines* 7(8), 1173–1183 (2008).

35 Sharma AK, Sharma MK. Plants as bioreactors: recent developments and emerging opportunities. *Biotechnol. Adv.* 27(6), 811–832 (2009).

36 Rybicki EP. Third International Conference on Plant-Based Vaccines and Antibodies. *Expert Rev. Vaccines* 8(9), 1151–1155 (2009).

37 Rybicki EP. Plant-made vaccines for humans and animals. *Plant Biotech. J.* 8(5), 620–637 (2010).

38 Rose RC, Lane C, Wilson S, Suzich JA, Rybicki EP, Williamson A-L. Oral vaccination of mice with human papillomavirus virus-like particles induces systemic virus-neutralizing antibodies. *Vaccine* 17(17), 2129–2135 (1999).

39 Gerber S, Lane C, Brown DM *et al.* Human papillomavirus virus-like particles are efficient oral immunogens when coadministered with *Escherichia coli* heat-labile enterotoxin mutant R192G or CpG DNA. *J. Virol.* 75 (10), 4752–4760 (2001).

40 Thönes N, Müller M. Oral immunization with different assembly forms of the HPV 16 major capsid protein L1 induces neutralizing antibodies and cytotoxic T-lymphocytes. *Virology* 369(2), 375–388 (2007).

41 Varsani A, Williamson AL, Rose RC, Jaffer M, Rybicki EP. Expression of human papillomavirus type 16 major capsid protein in transgenic *Nicotiana tabacum* cv. Xanthi. *Arch. Virol.* 148(9), 1771–1786 (2003).

42 Warzecha H, Mason HS, Lane C *et al.* Oral immunogenicity of human papillomavirus-like particles expressed in potato. *J. Virol.* 77(16), 8702–8711 (2003).

43 Biemelt S, Sonnewald U, Galmbacher P, Willmitzer L, Müller M. Production of human papillomavirus type 16 virus-like particles in transgenic plants. *J. Virol.* 77(17), 9211–9220 (2003).

44 Liu HL, Li WS, Lei T *et al.* Expression of human papillomavirus type 16 L1 protein in transgenic tobacco plants. *Acta Biochim. Biophys. Sin (Shanghai).* 37(3), 153–158 (2005).

45 Varsani A, Williamson AL, Stewart D, Rybicky EP. Transient expression of human papillomavirus type16 L1 protein in *Nicotiana benthamiana* using an infectious tobamovirus vector. *Virus Res.* 120 (1–2), 91–96 (2006).

46 Palmer KE, Benko A, Doucette SA *et al.* Protection of rabbits against cutaneous papillomavirus infection using recombinant tobacco mosaic virus containing L2 capsid epitopes. *Vaccine* 24(26), 5516–5525 (2006).

47 Pastrana DV, Buck CB, Pang YY *et al.* Reactivity of human sera in a sensitive, high-throughput pseudovirus-based papillomavirus neutralization assay for HPV16 and HPV18. *Virology* 321(2), 205–216 (2004).

48 Kohl T, Hitzeroth II, Stewart D *et al.* Plant-produced cottontail rabbit papillomavirus L1 protein protects against tumor challenge: a proof-of-concept study. *Clin. Vaccine Immunol.* 13(8), 845–853 (2006).

49 Kohl TO, Hitzeroth II, Christensen ND, Rybicki EP. Expression of HPV-11 L1 protein in transgenic *Arabidopsis thaliana* and *Nicotiana tabacum. BMC Biotechnol.* 7, 56 (2007).

50 Fischer R, Stoger E, Schillberg S, Christou P, Twyman RM. Plant-based production of biopharmaceuticals. *Curr. Opin. Plant Biol.* 7, 152–158 (2004).

51 Maclean J, Koekemoer M, Olivier AJ *et al.* Optimization of human papillomavirus type 16 (HPV-16) L1 expression in plants: comparison of the suitability of different HPV-16 *L1* gene variants and different cell-compartment localization. *J. Gen. Virol.* 88(5), 1460–1469 (2007).

52 Regnard GL, Halley-Stott RP, Tanzer FL, Hitzeroth II, Rybicki EP. High level protein expression in plants through the use of a novel autonomously replicating geminivirus shuttle vector. *Plant Biotechnol. J.* 8(1), 38–46 (2010).

53 Lenzi P, Scotti N, Alagna F *et al.* Translational fusion of chloroplast-expressed human papillomavirus type 16 L1 capsid protein enhances antigen accumulation in transplantomic tobacco. *Transgenic Res.* 17(6), 1091–1102 (2008).

54 Fernández-San Millán A, Ortigosa SM, Hervás-Stubbs S *et al.* Human papillomavirus L1 protein expressed in tobacco chloroplasts self-assembles into virus-like particles that are highly immunogenic. *Plant Biotechnol. J.* 6(5), 427–441 (2008).

55 Pereira R, Hitzeroth II, Rybicki EP. Insights into the role and function of L2, the minor capsid protein of papillomaviruses. *Arch. Virol.* 154(2), 187–197 (2009).

56 Jagu S, Karanam B, Gambhira R *et al.* Concatenated multitype L2 fusion proteins as candidate prophylactic pan-human papillomavirus vaccines. *J. Natl Cancer Inst.* 101(11), 782–792 (2009).

57 Slupetzky K, Gambhira R, Culp TD *et al.* A papillomavirus-like particle (VLP) vaccine displaying HPV16 L2 epitopes induces cross-neutralizing antibodies to HPV11. *Vaccine* 25(11), 2001–2010 (2007).

58 McCormick AA, Reddy S, Reinl SJ *et al.* Plant-produced idiotype vaccines for the treatment of non-Hodgkin's lymphoma: safety and immunogenicity in a Phase I clinical study. *Proc. Natl Acad. Sci. USA* 105(20), 10131–10136 (2008).

59 Frazer HI. Prevention of cervical cancer through papillomavirus vaccination. *Nat. Rev. Immunol.* 4, 46–54 (2004).

60 Franconi R, Di Bonito P, Dibello F *et al.* Plant-derived human papillomavirus 16 E7 oncoprotein induces immune response and specific tumour protection. *Cancer Res.* 62, 3654–3658 (2002).

61 Roggero P, Ciuffo M, Benvenuto E, Franconi R. The expression of a single-chain Fv antibody fragment in different plant hosts and tissues by using potato virus X as a vector. *Protein Expr. Purif.* 22(1), 70–74 (2001).

62 Dalsaard K. Isolation of a substance from *Quillaja saponaria* Molina with adjuvant activity in food-and mouth disease vaccines. *Arch. Gesamte Virusforsch.* 44, 243–254 (1974).

63 Feltkamp MC, Smits HL, Vierboom MP *et al.* Vaccination with cytotoxic T lymphocyte epitope-containing peptide protects against a tumor induced by human papillomavirus type16-transformed cells. *Eur. J. Immunol.* 23, 2242–2249 (1993).

64 Franconi R, Massa S, Illiano E *et al.* Exploiting the plant secretory pathway to improve the anticancer activity of a plant-derived HPV16 E7 vaccine. *Int. J. Immunopathol. Pharmacol.* 19(1), 187–197 (2006).

65 Di Bonito P, Grasso F, Mangino G *et al.* Immunomodulatory activity of a plant extract containing human papillomavirus 16-E7 protein in human monocyte-derived dendritic cells. *Int. J. Immunopathol. Pharmacol.* 22(4), 967–978 (2009).

66 Smahel M, Sima P, Ludvikova V, Vonka V. Modified HPV16 *E7* genes as DNA vaccine against E7-containing oncogenic cells. *Virology* 281(2), 231–238 (2001).

67 Musiychuk K, Stephenson N, Bi H *et al.* A launch vector for the production of vaccine antigens in plants. *Influenza Other Resp. Viruses* 1(1), 19–25 (2007).

68 Massa S, Franconi R, Brandi R *et al.* Anti-cancer activity of plant-produced HPV16 E7 vaccine. *Vaccine* 25, 3018–3021 (2007).

69 Venuti A, Massa S, Mett V *et al.* An E7-based therapeutic vaccine protects mice against HPV16 associated cancer. *Vaccine* 27, 3395–3397 (2009).

70 Skarjinskaia M, Karl J, Araujo A *et al.* Production of recombinant proteins in clonal root cultures using episomal expression vectors. *Biotechnol. Bioeng.* 100(4), 814–819 (2008).

71 Massa S, Skarjinskaia M, Mett V, Venuti A, Yusibov V, Franconi R. Plant platforms for producing anti-cancer therapeutic vaccines. Presented at: *Third International Conference on Plant-Based Vaccines and Antibodies.* University of Verona, Verona, Italy, 15–17 June 2009.

72 Torrent M, Llompart B, Lasserre-Ramassamy S *et al.* Eukaryotic protein production in designed storage organelles. *BMC Biol.* 7, 5 (2009).

73   Ohlschläger P, Pes M, Osen W *et al.* An improved rearranged human papillomavirus type 16 E7 DNA vaccine candidate (HPV-16 E7SH) induces an E7 wild type-specific T cell response. *Vaccine* 24(15), 2880–2893 (2006).

74   Savelyeva N, Munday R, Spellenberg MB, Lomonossoff GP, Stevenson FK. Plant viral genes in DNA idiotypic vaccines activate linked CD4$^+$ T-cell mediated immunity against B-cell malignancies. *Nat. Biotech.* 19, 760–764 (2001).

75   Savelyeva N, Zhu D, Stevenson FK. Engineering DNA vaccines that include plant virus coat proteins. *Biotechnol. Genet. Eng. Rev.* 20, 101–114 (2003).

76   Massa S, Simeone P, Muller A, Benvenuto E, Venuti A, Franconi R. Antitumor activity of DNA vaccines based on the human papillomavirus-16 E7

protein genetically fused to a plant virus coat protein. *Hum. Gene Ther.* 19(4), 354–364 (2008).

77   Lin KY, Guarnieri FG, Staveley-O'Carroll KF *et al.* Treatment of established tumors with a novel vaccine that enhances major histocompatibility class II presentation of tumor antigen. *Cancer Res.* 56, 21–26 (1996).

78   Morgenfeld M, Segretin ME, Wirth S. Potato virus X coat protein fusion to human papillomavirus 16 E7 oncoprotein enhance antigen stability and accumulation in tobacco chloroplast. *Mol. Biotechnol.* 43, 243–249 (2009).

79   Stirpe F, Battelli MG. Ribosome-inactivating proteins: progress and problems. *Cell Mol. Life Sci.* 63(16), 1850–1866 (2006).

80   Cerovská N, Hoffmeisterová H, Pecenková T *et al.* Transient expression of HPV16 E7 peptide (aa 44–60) and HPV16

L2 peptide (aa 108–120) on chimeric potyvirus-like particles using potato virus X-based vector. *Protein Exp. Purif.* 58(1), 154–161 (2008).

81   Paz De la Rosa G, Monroy-García A, Mora-García ML *et al.* An HPV 16 L1-based chimeric human papillomavirus-like particles containing a string of epitopes produced in plants is able to elicit humoral and cytotoxic T-cell activity in mice. *Virol. J.* 6, 1–11 (2009).

Patents

101   Sohn U, Nam HG, Park DH, Kim KH. Recombinant human papillomavirus vaccine expressed in transgenic plants. *United States Patent* 6444805 (2002).

102   Franconi R, Spanò L, Venuti A, Massa S. Vaccines based on genetic chimeras of viral and/or tumor antigens and/or and plant proteins. *Italian Patent* RM2009A000383 (2009).