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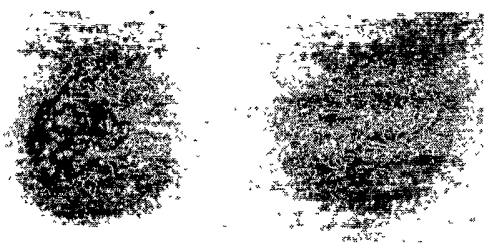
(54) Title: VECTORS, CONSTRUCTS, AND TRANSGENIC PLANTS FOR HPV-11 AND HPV-16 L1 CAPSID PROTEIN



(57) Abstract: Vectors and/or constructs of human papillomavirus genes are used in transforming host cells, such as bacteria, yeast cells, fungal cells, insect cells, mammalian cells and plant cells. The host cells are cultivated under conditions which permit spotaeousamly of at least one protein fragment of a protein encoded by the vector and/or gene construct into an immunogenic-response elicitig virus-like particle directed against human papillomavirus.

VECTORS, CONSTRUCTS, AND TRANSGENIC PLANTS FOR HPV-11 AND HPV-16 L1 CAPSID PROTEIN

BACKGROUND OF THE INVENTION

The present invention relates to vectors and/or constructs, and transgenic organisms.

In this specification the following terms, phrases and/or clauses are to be understood to mean:

"Construct" - as used herein is synonymous with terms such as "conjugate", "cassette", and "hybrid" and includes a nucleotide sequence directly or indirectly linked to a promoter. The construct may contain or express a marker, which allows for the selection for the construct in a host cell.

"Expression vector" - as used herein means a construct capable of *in vivo* or *in vitro* expression.

"Expression" is understood to mean the production of a protein from a DNA template via transcription and translation.

"Genetically modified organism" - as used herein refers to an organism into which a nucleotide sequence has been introduced as a consequence of human intervention in a way that did not occur naturally through mating or natural recombination or both.

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"*In silico*" - parts of assays and/or processes described herein may be performed by use of suitable computational software. Such assays and/or processes so performed shall be understood to be encompassed herein.

"Nucleotide sequence" - as used herein is synonymous with the term "nucleotide acid sequence" and/or the term "polynucleic acid" and/or the term "polynucleotide" and includes genomic DNA, cDNA, recombinant DNA, synthetic DNA, and RNA, and any combinations of the afore-mentioned, also the nucleotide sequence may be double-stranded or single-stranded whether representing the sense or the antisense strand. Preferably, the term "nucleotide sequence" means DNA.

"Operably linked" - as used herein refers to a juxtaposition wherein the promoter which is "operably linked" to a coding sequence is ligated in such manner that expression of the coding sequence is achieved under conditions compatible with the control sequences (i.e. *inter alia* promoters, enhancers and other expression regulation signals).

"Promoter" - as used herein is a nucleotide sequence that directs the regulated transcription of a nucleotide sequence.

"Protein" - as used herein is intended to denote both peptides and polypeptides.

"Recombinant organism" - as used herein refers to a transgenic organism containing a vector and/or construct expressing a nucleotide sequence.

"Transgenic organism"- as used herein includes a genetically modified organism that comprises the vector and/or construct, according to the invention, wherein a promoter can allow expression of a nucleotide sequence, or parts thereof, of the vector and/or construct according to the invention within the organism.

"Vector" - as used herein includes expression vectors, replicable vectors, transformation vectors, shuttle vectors, cosmids, plasmids, phages, viruses and yeast artificial chromosomes or any combination thereof.

"VLP" - as used herein refers to virus-like particles.

Papillomaviruses (PVs) belong to the taxonomic family *Papillomaviridae*, and are small, non-enveloped, double-stranded (ds) DNA viruses. These viruses infect a wide range of higher vertebrates and are highly species-specific. Upon infection, human papillomaviruses (HPVs) have a particular tropism for undifferentiated squamous epithelial cells and apart from being associated with plantar warts, flat warts, and genital warts (condyloma acuminata). HPV infections are also linked to a disease called epidermodysplasia verruciformis, a rare lifelong disease characterized by disseminating papillomas. There has, furthermore, been an epidemiological and biochemical link of certain papillomavirus isolates with urogenital cancers, including cervical, vulvar, penile cancer, and malignant transformation of epidermodysplasia verruciformis lesions. Of the characterized HPV types, about 27 have been identified as the causal agents of anogenital infections. HPV types 6, 11, 16, 18, 31, 33, 35 and 42 have been identified as being the most prevalent, whereas types 16, 18, 31, 33, 51 and 54 have been associated with anogenital carcinomas, believed to be of high risk, more particularly HPV-16 has been found in 95% of cervical cancers.

Until recently it was believed that high-risk genital HPVs were only transmitted during sexual intercourse, however studies indicate that infants acquire high-risk HPV infections from their mothers at birth. In addition to non-sexual parent-to-child transmission, investigators detected HPV DNA in vaginal swabs from women, in cervical-vaginal specimens from young girls, and in vulval swabs from 9 out of 61 women who claimed no history of sexual contact.

Initial HPV infection causes cervical intraepithelial neoplasia (CIN), more commonly known as pre-cancerous lesions. It has been found that in some women there is a spontaneous regression of CIN, but this is not always the case and lesions can persist for years, increasing the likelihood of progression towards cervical cancer.

Treatment methods ranging from cryotherapy, surgical excision and topical treatments do not often alleviate problems and for patients who do respond to the treatment, recurrence is often a problem. Ultimate destruction of all infected tissue does not appear to be feasible, since multifocal disease and latency are common features of the infections. Antiviral drugs designed to target and arrest viral replication seem to be ineffective in the case of HPV infections, firstly because the virus does not replicate in the cells that maintain the infection and secondly due to the fact that viral replication genes seem to get lost through integration, rearrangement and deletions.

Traditionally most prophylactic vaccines have consisted of live, attenuated virus or formalin-inactivated virus. Due to the difficulties and risks involved in generating large quantities of these traditional vaccines there has been great emphasis to develop a viral protein subunit vaccine and a method for the production thereof.

SUMMARY OF THE INVENTION

In accordance with the invention there is provided a vector and/or construct selected from the group consisting of:

a. HPV 11 L1 NLS^-;
b. HPV 11 L1;
c. HPV 16 L1 NLS^-;
d. HPV16 L1;
e. pART7;
f. pART27;

in particular the group consisting of:

(i) HPV 11 OR HPV 16 L1 GENE (504aa);
(ii) HPV 11 OR HPV 16 L1 gene lacking nuclear localisation signal (NLS-, Δ483);
(iii) HPV 11 OR HPV 16 L1 gene lacking 10 N-terminal codons (ΔN10);
(iv) HPV 11 OR HPV 16 L1 gene lacking 10 N-terminal codons and NLS (ΔN10Δ483);
(v) HPV 11 OR HPV 16 L1 gene with a C to G mutation (pen) at aa428 (pen504);
(vi) HPV 11 or HPV 16 L1 genes with pen and ΔN10 modifications;
(vii) HPV 11 or HPV 16 L1 genes with pen and Δ483 modifications; and
(viii) HPV 11 or HPV 16 L1 genes with pen and ΔN10Δ483 modifications.

Furthermore, in accordance with the invention there is provided the use of the vector and/or construct in a method of transforming a host cell.

Still furthermore, in accordance with the invention there is provided a host cell transformed with the vector and/or construct wherein the transformed host cell is preferably:

a. a bacterium;
b. a yeast cell;
c. a fungal cell;
d. an insect cell;
e. a mammalian cell;
f. a plant cell,

cultivated under conditions which permit spontaneous assembly of at least one protein or fragment of protein encoded by the vector and/or construct

into an immunogenic-response eliciting virus-like particle directed against human papillomavirus, more particularly human papillomavirus-11 and/or human papillomavirus-16, most preferably the vector and/or construct is stably integrated into a host-cell genome.

Still furthermore, in accordance with the invention there is provided a non-human multicellular transgenic organism transformed by the vector and/or construct cultivated under conditions which permit spontaneous assembly of at least one protein or fragment of a protein encoded by the vector and/or construct into an immunogenic-response eliciting virus-like particle directed against human papillomavirus, more particularly human papillomavirus-11 and/or human papillomavirus-16, preferably the transgenic organism is selected from the group consisting of:

1. *Nicotiana tabacum cv* Xanthi;
2. *Nicotiana tabacum cv* Soulouk;
3. *Lycopersicon esculentum* cv Tiny Tom; and
4. *Arabidopsis thaliana* cv Columbia.

Furthermore, the invention provides for systemic expression of the protein or fragment of the protein by the transgenic organism.

BRIEF DESCRIPTION OF THE DRAWINGS

Features of this invention will become apparent from the following description of certain embodiments of the invention described below, by way of example only, and with reference to the accompanying drawings in which:

Figure 1 shows a plasmid map of pART7;

Figure 2 shows a plasmid map of pART27;

Figure 3 shows an electron micrograph of HPV-16 VLPs isolated from transgenic tobacco, trapped with HPV-16-specific MAb; and

Figure 4 shows an electron micrograph of HPV-11 VLPs isolated from transgenic *Arabidopsis*.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS OF THE INVENTION

The methods for preparing transgenic organisms are known to those skilled in the art and will not be developed in depth in the present description.

Cloning and preparation of *Agrobacterium* clones

The HPV-11 L1 and HPV-16 L1 gene fragments were individually cloned into the multiple cloning site (MCS) of the primary vector pART7 using the *HIND* III and *EcoR* I restriction sites to give the constructs HPV 11 L1 NLS^-, HPV 11 L1, HPV 16 L1 NLS^-, and HPV 16 L1.

The complete cassette from the pART7 vector (Figure 1) was excised by *Not* I digestion and cloned into the binary vector pART27 (Figure 2).

The respective pART27 clone was transfected into *Agrobacterium tumefaciens* strain C58C1.

Leaf disc transformation and tissue culture

Nicotiana tabacum cv Xanthi and Soulouk leaf discs were transformed with the *Agrobacterium* clone and transformants were selected on kanamycin tissue culture media as described by Turpen et al. (Transfection of whole plants from wounds inoculated with *Agrobacterium tumefaciens* containing cDNA of tobacco mosaic virus. J. Virol. Meth. 42: 227-240).

Lycopersicon esculentum cv Tiny Tom cotyledons were transformed with the *Agrobacterium* clone as described by Pfitzner (Transformation of tomato. J. Mol. Biol. Meth. 81:359-363).

Flowering *Arabidopsis thaliana* cv Columbia plants were transformed with the *Agrobacterium* clone as described by Clough and Bent (Simplified *Arabidopsis* Transformation Protocol available at http://www.cropsci.uiuc.edu/~a-bent/protocol.hmtl) and transformants were selected on kanamycin tissue culture media as described by Turpen et al. (Transfection of whole plants from wounds inoculated with *Agrobacterium tumefaciens* containing cDNA of tobacco mosaic virus. J. Virol. Meth. 42: 227-240).

Screening of transgenic plants

Plant genomic DNA from transformants was extracted using the stated method of Dellaporta et al. (A plant DNA minipreparation: Version II. Cold Spring Harbour Laboratory. Cold Spring Harbour) and the presence of the integrated gene was confirmed by PCR.

Extraction of VLPs from transgenic plants

The plant leaf material was homogenised in 1:2 volumes (plant material: buffer) of buffer (for HPV use PBS with 0.5M NaCl) and pressed out through cheesecloth over funnel until the pulp was dry. The extract was centrifuged for 10 minutes at 4°C (3000rpm). 10% PEG (MW 8000) was added to the supernatant and left to dissolve overnight at 4°C. This was then centrifuged at 3000rpm for 10 minutes at 4°C. The pellet was resuspended overnight at 4°C in 1/10th the original volume of PBS (0.5M NaCl). The mixture was then centrifuged at 3000 rpm for 15 minutes in order to remove all denatured non-soluble components. The extract was overlaid onto a 40% sucrose cushion (made in PBS with 0.5M NaCl) and centrifuged at 100 000 x g (for Sorvall SW28, 24 000 rpm) at 10°C for 2.5

hours. The pellet was resuspended in a small quantity of PBS (0.5 M NaCl) and the suspension was passed through 18 & 26 gauge needles to reduce its viscosity. This suspension was loaded onto a linear sucrose gradient of 10-40% and spun at 100 000 x g (for SW 28, 24 000 rpm) at 10°C for 3 hours. The bands observed under scattered light were removed with a needle and syringe and dialyzed against PBS (0.5M NaCl) at 4°C for 12-24 hours.

Electron microscope analysis

The VLPs were trapped onto copper grids coated with polyclonal antibody (specific for HPV-11 L1) R399 and negatively stained with 2% uranyl acetate. The grids were viewed under the 200CX electron microscope.

The L1 gene was stably integrated into the *Nicotiana tabacum cv* Xanthi and Soulouk genomes in the R_0 (first transgenic lines). A general 3:1 Mendelian inheritance was observed for the L1 gene for the self-pollinated transgenic plants in the T_1 generation (first generation). Furthermore the L1 gene was detected in the T_2 (second generation) through to the T_5 (fifth generation) in transgenic *Arabidopsis thaliana* plants.

The VLPs observed under the electron microscope (trapped or untrapped with antibody) were similar in appearance to those resulting from L1 gene expression using the baculovirus insect cell system. The size of the particles was observed to be approximately 50-60nm in diameter (*Arabidopsis thaliana*) and partially degraded particles were observed upon examination of *Nicotiana tabacum* protein extract.

Expression of Papillomavirus Genes in Transgenic Plants

In further studies, transgenic lines containing HPV-16 L1 genes, which are now in at least the second generation of selection, were made. In addition, both *Arabidopsis thaliana* and *Nicotiana tabacum* plants were transformed with HPV-11 NLS- L1 genes. Tobacco plants have also recently been

transformed with CRPV L1. All of these plant lines were shown to produce VLPs, albeit at low concentrations. The inclusion or absence of NLS sequences appears to make little difference for HPV-16 L1 VLP expression, although HPV-11 L1 may be toxic if localised to plant cell nuclei. The HPV-16 VLPs were trapped by V5 MAb, as shown in Figure 3 (Bar = 70nm), indicating that they are antigenically appropriate for vaccine use.

HPV-11 VLPs isolated from transgenic *Arabidopsis* are shown in figure 4 (Bar = 70nm).

An HPV-11 L1 lacking a nuclear localisation signal (NLS-, Δ483) protein and a number of different HPV-16 L1 and L1 NLS- protein constructs have been expressed in transgenic plants, and it has been determined that these make T=7 icosahedral particle VLPs, are immunogenic in rabbits upon injection, and appear morphologically identical to VLPs made in a baculovirus expression system in insect cells. Additionally, it has been determined that HPV-16 VLPs made in transgenic plants bind a monoclonal antibody (MAb V5) which is conformationally specific and blocks neutralising antibody binding. These findings indicate that VLPs made in plants are functionally identical to VLPs made in insect cells. Further, the following constructs have been expressed via recombinant baculoviruses in insect cells, which bind the same conformationally-specific and sequence-specific MAbs as are bound by unaltered L1 protein:

a. HPV 16 L1 gene lacking the 10 N-terminal codons (ΔN10), which assembles into T=1 and also T=7 icosahedral particles;
b. HPV 16 L1 gene lacking 10 N-terminal codons and NLS (ΔN10Δ483), which assembles into T=1 and also T=7 icosahedral particles;
c. HPV 16 L1 gene with a C to G mutation (pen) at aa428 (pen504), which assembles into pentamers;
d. HPV 16 L1 gene with pen and ΔN10 modifications, which assembles into pentamers;

e. HPV 16 L1 gene with pen and Δ483 modifications, which assembles into pentamers; and

f. HPV 16 L1 gene with pen and ΔN10Δ483 modifications, which assembles into pentamers.

It is apparent to one skilled in the art that these constructs could be expressed in transgenic plants and should behave in exactly the same way.

It will be immediately apparent to a person skilled in the art that although certain embodiments only of the invention have been set out herein, other modifications and/or variations of the invention are possible. Such modifications and/or variations are to be considered as falling within the scope of the present invention.

CLAIMS

1. A vector and/or a gene construct selected from the group consisting of:

   a. HPV11 L1 NLS$^-$;
   b. HPV 11 L1;
   c. HPV 16 L1 NLS$^-$;
   d. HPV16 L1;
   e. pART7; and
   f. pART27.

2. A vector and/or a gene construct according to claim 1 selected from the group consisting of:

   (i) HPV 11 OR HPV 16 L1 GENE (504aa);
   (ii) HPV 11 OR HPV 16 L1 gene lacking nuclear localisation signal (NLS-, Δ483);
   (iii) HPV 11 OR HPV 16 L1 gene lacking 10 N-terminal codons (ΔN10);
   (iv) HPV 11 OR HPV 16 L1 gene lacking 10 N-terminal codons and NLS (ΔN10Δ483);
   (v) HPV 11 OR HPV 16 L1 gene with a C to G mutation (pen) at aa428 (pen504);
   (vi) HPV 11 or HPV 16 L1 genes with pen and ΔN10 modifications;
   (vii) HPV 11 or HPV 16 L1 genes with pen and Δ483 modifications; and
   (viii) HPV 11 or HPV 16 L1 genes with pen and ΔN10Δ483 modifications.

3. The use of a vector and/or a gene construct of either of claims 1 or 2 in a method of transforming a host cell.

4. A host cell transformed with a vector and/or the gene construct of either of claims 1 or 2, which is selected from the group comprising a bacterium, a yeast cell, a fungal cell, an insect cell, a mammalian cell, and a plant cell.

5. A host cell according to claim 4, which is cultivated under conditions which permit spontaneous assembly of at least one protein or fragment of protein encoded by the vector and/or gene construct into an immunogenic-response eliciting virus-like particle directed against human papillomavirus.

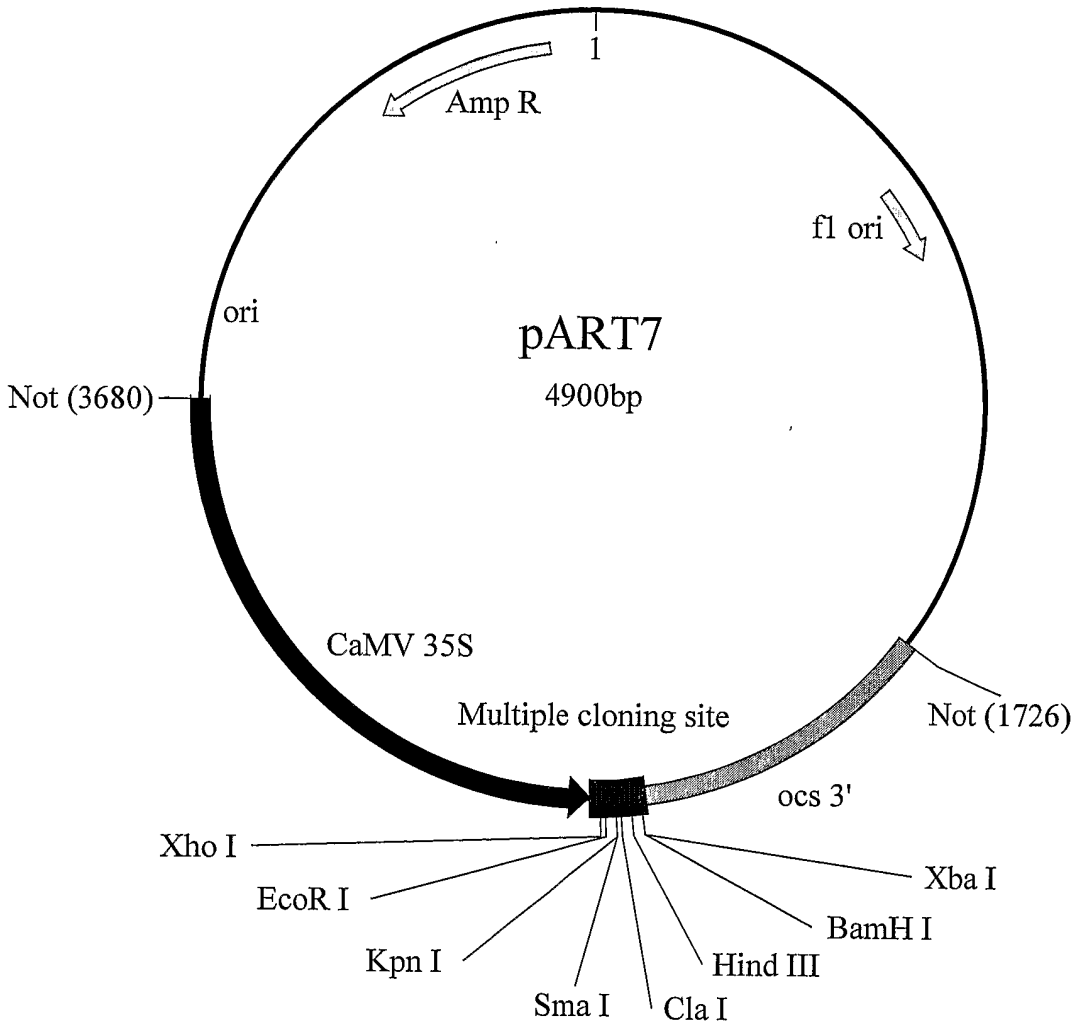
6. A host cell according to claim 5, wherein the human papillomavirus is human papillomavirus-11 and/or human papillomavirus-16.

7. A host cell according to claim 6, wherein the vector and/or the gene construct is stably integrated into a host-cell genome.

8. A non-human multicellular transgenic organism, which is transformed by the vector and/or the gene construct of either of claims 1 or 2 cultivated under conditions which permit spontaneous assembly of at least one protein or fragment of a protein encoded by the vector and/or the gene construct into an immunogenic-response eliciting virus-like particle directed against human papillomavirus.

9. A transgenic organism according to claim 8, wherein the virus-like particle is directed against human papillomavirus-11 and/or human papillomavirus-16.

10. A transgenic organism according to claim 9, which is selected from the group consisting of *Nicotiana tabacum cv* Xanthi, *Nicotiana tabacum cv* Soulouk, *Lycopersicon esculentum* cv Tiny Tom; and *Arabidopsis thaliana* cv Columbia.


1
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f1 ori
ori
pART7
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CaMV 35S
Multiple cloning site
Not (1726)
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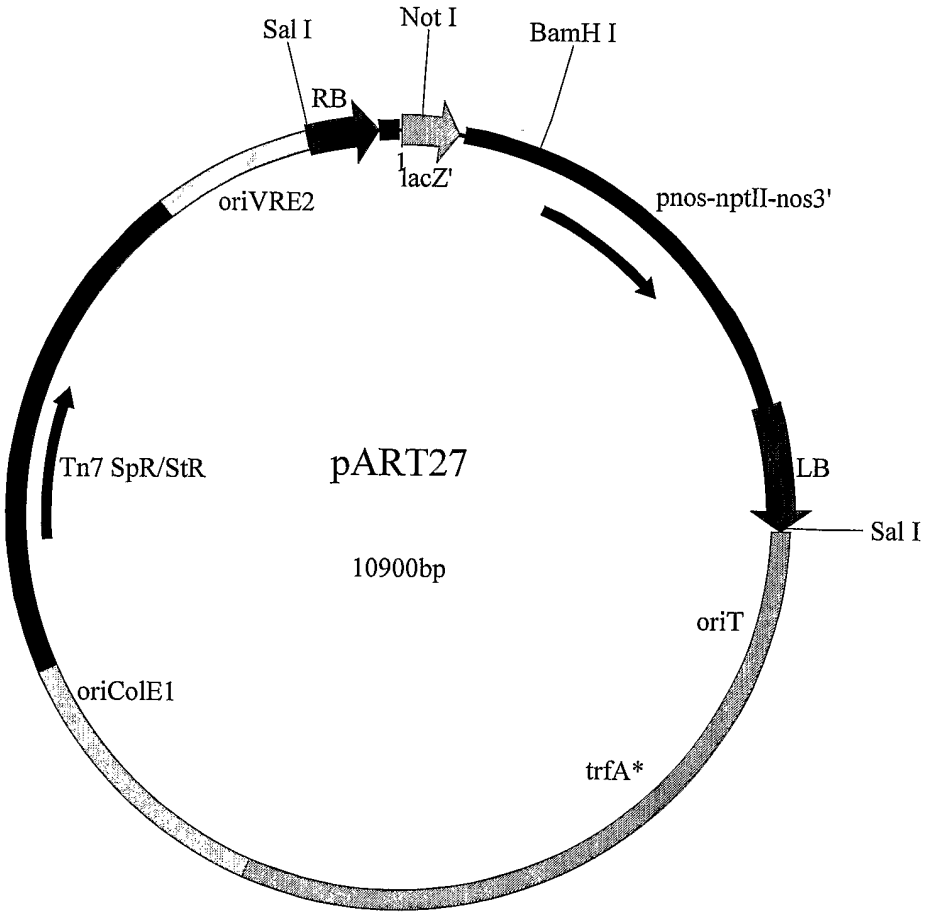
Xho I
EcoR I
Kpn I
Sma I
Cla I
Hind III
BamH I
Xba I

FIGURE 1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BamH I
RB
lacZ'
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pnos-nptII-nos3'
Tn7 SpR/StR
pART27
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LB
Sal I
oriT
oriColE1
trfA*

FIGURE 2

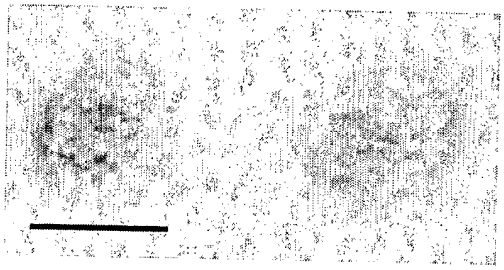

FIGURE 3

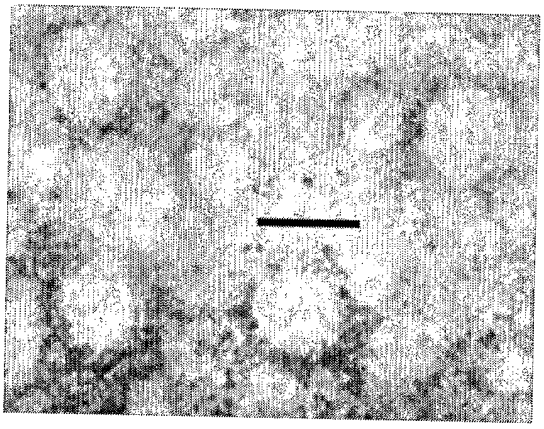

FIGURE 4

INTERNATIONAL SEARCH REPORT

Inte ıl Application No
PCT/IB 02/03532

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/025 C12N15/82 C12N5/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
BIOSIS, EPO-Internal, MEDLINE, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MERLE ERIC ET AL: "Nuclear import of HPV11 L1 capsid protein is mediated by karyopherin alpha2beta1 heterodimers." JOURNAL OF CELLULAR BIOCHEMISTRY, vol. 74, no. 4, pages 628-637, XP002216858 ISSN: 0730-2312 table 1 ---	1-7
X	CHEN XIAOJIANG S ET AL: "Structure of small virus-like particles assembled from the L1 protein of human papillomavirus 16." MOLECULAR CELL, vol. 5, no. 3, March 2000 (2000-03), pages 557-567, XP002216859 ISSN: 1097-2765 page 557, column 1, paragraph 1 --- -/--	1-7

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

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Date of the actual completion of the international search	Date of mailing of the international search report
7 November 2002	20/11/2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Friedrich, C

INTERNATIONAL SEARCH REPORT

Inte al Application No
PCT/IB 02/03532

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	ZHOU J ET AL: "IDENTIFICATION OF THE NUCLEAR LOCALIZATION SIGNAL OF HUMAN PAPILLOMAVIRUS TYPE 16 L1 PROTEIN" VIROLOGY, vol. 185, no. 2, 1991, pages 625-632, XP001118208 ISSN: 0042-6822 page 629; table 2 ---	1-7
X	ZHOU J ET AL: "EXPRESSION OF VACCINIA RECOMBINANT HPV 16 L1 AND L2 ORF PROTEINS IN EPITHELIAL CELLS IS SUFFICIENT FOR ASSEMBLY OF HPV VIRION-LIKE PARTICLES" VIROLOGY, RAVEN PRESS, NEW YORK, NY, US, vol. 185, no. 1, November 1991 (1991-11), pages 251-257, XP000857171 ISSN: 0042-6822 the whole document ---	1-7
P,X	WO 02 38769 A (DEUTSCHES KREBSFORSCH ;IPK INST FUER PFLANZENGENETIK (DE); SONNEWA) 16 May 2002 (2002-05-16) examples 6,7 ---	1-10
X	GLEAVE A P: "A CERSATILE BINARY VECTOR SYSTEM WITH A T-DNA ORGANISATIONAL STRUCTURE CONDUCIVE TO EFFICIENT INTEGRATION OF CLONED DNA INTO THEPLANT GENOME" PLANT MOLECULAR BIOLOGY, NIJHOFF PUBLISHERS, DORDRECHT, NL, vol. 20, 1992, pages 1203-1207, XP002043805 ISSN: 0167-4412 the whole document ---	1,3-10
A	LUDMERER STEVEN W ET AL: "HPV11 mutant virus-like particles elicit immune responses that neutralize virus and delineate a novel neutralizing domain." VIROLOGY., vol. 266, no. 2, 20 January 2000 (2000-01-20), pages 237-245, XP002216860 ISSN: 0042-6822 the whole document ---	1-10

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	NARDELLI-HAEFLIGER D ET AL: "HUMAN PAPILLOMAVIRUS TYPE 16 VIRUS.LIKE PARTICLES EXPRESSED IN ATTENUATED SALMONELLA TYPHIMURIUM ELICIT MUCOSAL AND SYSTEMIC NEUTRALIZING ANTIBODIES IN MICE" INFECTION AND IMMUNITY, AMERICAN SOCIETY FOR MICROBIOLOGY. WASHINGTON, US, vol. 65, no. 8, 1 August 1997 (1997-08-01), pages 3328-3336, XP002054900 ISSN: 0019-9567 the whole document	1-10
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INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. Application No
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		WO 0238769 A2	16-05-2002