

Production of vaccines for treatment of infectious diseases by transgenic plants

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ABSTRACT

Since the first pathogen antigen was expressed in transgenic plants with the aim of producing edible vaccine in early 1990s, transgenic plants have become a well-established expression system for production of alternative vaccines against various human and animal infectious diseases. The main focus of plant expression systems in the last five years has been on improving expression of well-studied antigens such as porcine reproductive and respiratory syndrome (PRRSV), bovine viral diarrhoea disease virus (BVDV), foot and mouth disease virus (FMDV), hepatitis B surface antigen (HBsAg), rabies G protein, rotavirus, Newcastle disease virus (NDV), Norwalk virus capsid protein (NVCP), avian influenza virus H5N1, *Escherichia coli* heat-labile enterotoxin subunit B (LT-B), cholera toxin B (CT-B), human immunodeficiency virus (HIV), arteriosclerosis, ebola and anthrax. Significant increases in expression have been obtained using improved expression vectors, different plant species and transformation methods.

Key words: transgenic plants; vaccine production; recombinant proteins; antibodies; infectious diseases; risk assessment

IZVLEČEK

ZDRAVLJENJE NALEZLJIVIH BOLEZNI S CEPIVI PRIDOBLENIMI S TRANSGENIMI RASTLINAMI

Pridobivanje aktivnih sestavin za cepiva s transgenimi rastlinami se je začelo v devetdesetih letih prejšnjega stoletja. Od takrat se transgene rastline uveljavljajo kot alternativni ekspresijski sistem za sintezo aktivnih komponent za cepiva proti številnim humanim in živalskim nalezljivim boleznim. V zadnjih petih letih je bil glavni poudarek razvoja namenjen optimizaciji rastlinskih ekspresijskih sistemov za dobro proučene antigene, kot so virus prašičjega respiratornega in reproduktivnega sindroma, virus goveje virusne diareje, virus parkljevke, površinski antigen hepatitisa B, G protein virusa stekline, rotavirus, virus atipične kokoške kuge, plaščni protein Norwalk virusa, sev ptičje gripe H5N1, temperaturno nestabilna B podenota enterotoksina *Escherichia coli*, kolera toksin B, HIV, arterioskleroza, ebola in antraks. Uporaba optimiziranih transformacijskih metod in ekspresijskih vektorjev v kombinaciji s primerno izbrano rastlinsko vrsto je v večini naštetih primerov vodila do opaznega izboljšanja sinteze posameznih učinkovin.

Ključne besede: transgene rastline; pridobivanje cepiv; rekombinantni proteini; protitelesa; nalezljive bolezni; ocena tveganja

1 INTRODUCTION

Vaccination is the most effective known way of preventing outbreaks and spreading of viral and bacterial infections. Traditional vaccines contained attenuated or inactivated pathogens, however the development of molecular biology, genetics, medicine and biotechnology in past decades

resulted in many novel vaccine types and production systems. During the last 20 years, transgenic plants have started gaining importance as a potential alternative host system for expressing high amounts of safe, cheap, effective easily applicable oral vaccines. Some of the main

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advantages of plant-made vaccines are the absence of pathogens commonly found in mammalian, bacterial and yeast production systems, easier storage and transport and ability of inducing both systemic and mucosal immune response thus granting bigger protection against pathogen challenge. The main focus of the initial research as well as the recent development has been expression of various antigens belonging to agents of viral and bacterial infectious diseases of

humans, animals or both. Most of the expressed antigens have been successfully evaluated in animal and human models. Besides that quite a lot of research has been done in the field of expressing antigens related with autoimmune diseases, such as arteriosclerosis, diabetes type 1, rheumatoid arthritis and multiple sclerosis. The following article aims to cover some of the recent advances in expressing pathogen antigens in transgenic plants, transformation methods and vectors.

2 VIRAL VACCINES

2.1 Porcine reproductive and respiratory syndrome

Porcine reproductive and respiratory syndrome (PRRS) is a highly transmissible disease of sows and piglets, which was first reported in the US in 1987 and in Europe in 1990 (Chen and Liu, 2011) and has since spread to become one of the most devastating infectious diseases of swine industry nowadays, causing major economic losses in swine herds worldwide. Its cause is the porcine reproductive and respiratory syndrome virus (PRRSV). Currently, modified-live and inactivated (killed-virus) PRRSV vaccines are being used, but due to safety risks (reversion back to virulence) and inability of protecting against heterologous PRRSV infections of the first ones and weak effectiveness of the second ones, there's a great demand for development of novel vaccines (Chia et al., 2010; Hu et al., 2012; Renukaradhya et al., 2015). Porcine reproductive and respiratory syndrome GP5 glycoprotein 5 (PRRSV GP5), one of the main target antigens, was first expressed in transgenic tobacco plants in 2010, with expression levels reaching app. 0.011 % total soluble protein (TSP), despite the addition of endoplasmic reticulum (ER) retention signal KDEL and cauliflower mosaic virus (CaMV 35S) promoter. However, oral immunization with fresh leaf tissue triggered both PRRSV-specific humoral and cellular immune response (saliva IgA and systemic IgG) in mice (Chia et al., 2010). With the aim of increasing the expression level of GP5, Chia et al. (2011) later fused GP5 with *Escherichia coli* heat-labile toxin subunit B (LT-B) and additional endoplasmic reticulum targeting HDEL sequence, expressed it in *Nicotiana tabacum* under the control of ubiquitin promoter 1 (UBQ1). The

immunogenicity of the obtained fusion protein was evaluated in orally immunized pigs. Expression level in this case was 0.0155 % TSP. LT-B-GP5 fusion protein was able to induce GP5-specific PRRSV mucosal and systemic immunity after intranasal PRRSV challenge at slightly higher rate than GP5 alone. GP5 protein was also successfully expressed in transgenic potato tubers (yields 0.8 – 1.2 µg/g) and leaves (yields 2.5 – 4.7 µg/g) (Chen and Liu, 2011) and induced the production of PRRSV-specific intestine IgA and serum IgG when applied orally as crude tuber extracts. Chan et al. (2013) expressed GP5 in transgenic banana leaf tissue, driven by CaMV 35S promoter with 3'-flanking Mh-UBQ1 matrix-associated regions (MARs) and HDEL, with yields 0.021 – 0.037 % of TSP (154 – 257 ng/g of fresh banana leaves). That is 2-3 times higher than in transgenic tobacco (0.018 - 0.0155 % of TSP) (Chan et al, 2013), while the levels of neutralizing antibody (NA) titres in orally immunized pigs 3 weeks post infection (WPI) were similar to those obtained in pigs orally immunized with GP5 from transgenic tobacco- 1:4 - 1:16 (Chia et al., 2011). Immunized pigs developed PRRSV-specific humoral and cellular immune response. Hu et al. (2012) expressed PRRSV M protein driven by UBQ promoter in transgenic corn calli and obtained strong consecutive expression as well as induction of humoral, mucosal and cellular immune responses in orally immunized mice (30 mg of dried corn powder with adjuvants). The highest M protein yield was 5.1 µg/g of fresh and 86 µg/g of lyophilized callus. Recently, Piron et al. (2014) expressed a whole Eu-prototype LV PRRSV antigenic set in seeds of *Arabidopsis thaliana*, with or without transmembrane domains, with added affinity tags or stabilizing protein domains- Fc IgG

chain or green fluorescent protein (GFP), regulatory sequences of *Phaseolus vulgaris*, N-terminal fusion of *A. thaliana* 2S albumin seed storage signal peptide and C-terminal ER-retention signal HDEL. Even though all antigens were found to be correctly N-glycosylated, further studies will be needed to determine whether or not they have really been processed in ER. Fusion of stabilizing protein domains to truncated GP3- GP5-Tm positively affected their accumulation, the most of all GFP fusion, its addition to truncated GP3 and GP4 resulted in accumulation of 2.74 % and 2.36 %, respectively, which is way above the lowest economically feasible level. Full-length Gp4 and GP5 accumulated only at levels 0.08 and 0.1 % of TSP, respectively, while fusion of stabilizing protein domain increased it up to 2.36 % of TSP for GP3 and GP4. Fusion of pFc and mFc2a to truncated antigens increased the accumulation (from 0.05 % to 0.67 % and 1.59 %, respectively) while addition of pFc to GP5 decreased the accumulation (from 0.10 % to 0.01 % of TSP). Addition of Fc domain also allowed for single-step protein-A affinity chromatography with recovery of about 60 % (Piron et al., 2014). All 3 tested antigens (GFP-fused truncated GP3, mFc2a-fused truncated Gp4 and normal-size-mFc2a-fused GP4) induced high GP4-Tm-specific Ab titres, only GP4-Tm:mFc2a elicited NA. In pigs, there were no NA detected when immunized with pFc-fused truncated GP3, GP4 and GP5 and 5 weeks after the second immunization only 1 pig had high serum Ab titres. Recently, Uribe-Campero et al. (2015) co-expressed PRRSV GP5, M and N protein in *Nicotiana glauca* leaf tissue. *Agrobacterium*-mediated transformation was performed. The proteins assembled in virus-like particles (VLPs) of expected size (15, 19 and 25 kDa). When applied to mice by intraperitoneal immunization, the VLPs were shown to be immunogenic in mice.

2.2 Bovine viral diarrhea virus

Bovine viral diarrhea disease is an important cause of economic loss in global bovine herds, caused by bovine viral diarrhea virus (BVDV). Currently, inactivated vaccines are being used, however the E2 protein has been deemed the main target in developing novel subunit vaccines. Dus Santos and Widgorovitz (2005) produced transgenic alfalfa plants containing the gene for BVDV E2 glycoprotein under the control of CaMV 35S

promoter with TEV and KDEL signal sequences, transported into alfalfa in recombinant binary pBI121-TEV-Stag-E2-His-SEKDEL vector by *A. tumefaciens* transformation method. The approximate concentration of the recombinant protein expressed in 2 highest yielding plants was estimated between 0.05 and 0.5 mg/g TSP. In 2012, Nelson et al. expressed a truncated version of BVDV glycoprotein E2 (tE2), lacking the transmembrane domain with additional ER-retention signal KDEL, 2S2 *A. thaliana* plant secretory pathway signal, the Kozak sequence and a hexa-histidine tag, in transgenic tobacco plants (*N. tabacum*). pK7GW2 *Agrobacterium* binary vector was transported into leaf tissue by *Agrobacterium*-mediated transformation. The protein accumulated at levels app. 1.3 % TSP (20 µg/g) which is suitable for mass scale production and elicited an immune response comparable to that of commercial whole virus vaccine in subcutaneously immunized guinea pigs either with oily or aqueous adjuvant (86 % seroconversion with anti-BVDV Ab titres > 0.6 and 75 % seroconversion, respectively). When administered with aqueous adjuvant, recombinant tE2 elicited NA titers equal or higher than titers elicited by conventional vaccine. A year later, Aguirreburualde et al. (2013) expressed a truncated E2 protein, fused to APCH gene, under the control of Cassava striate mosaic virus (CsMV) promoter in recombinant binary plasmid pCs-APCHtE2, transformed into alfalfa petioles by *Agrobacterium*-mediated co-cultivation. APCH-tE2 was stably expressed in alfalfa leaves with level of 0.1 % of TSP, which is a significant increase as compared with the previous work. The final yield of 3.45 µg/ml of Ag permitted the formulation of an effective vaccine for cattle immunization. Parentally immunized guinea pigs developed NA titres > 2.4 and immunized cattle developed NA titres > 2 at the time of vaccination (t60). After challenge, 6 out of 8 bovines inoculated with 3 µg of alfalfa-produced APCH-tE2 showed complete virological protection after virus challenge. Aguirreburualde et al. (2013) suspect the APCH signal sequence directs the protein secretion to the apoplast but further investigations of this are needed. Average expression level of APCH-tE2 was 3 µg/ml of extract. From 50 g of transgene leaf tissue 80 µg of APCH-tE2 can be produced after processing,

which suffices for 27 doses of vaccine for cattle (1 dose = 3 µg of APCH-tE2).

2.3 Foot and mouth disease virus

Foot and mouth disease is a highly contagious disease of cloven-foot animals and a major cause of economical loss in livestock. There are 7 different serotypes of the foot and mouth disease virus (FMDV), infection with any serotype doesn't confer immunity against the others (Zhang et al., 2011). Currently used attenuated and conventionally inactivated vaccines are not completely safe neither effective so there is a great focus in developing novel vaccines. Experimental vaccines containing FMDV antigens from transgenic plants have been reported safer than conventional vaccines; they are also cheaper and more effective. The first experimental plant-made FMDV vaccine was produced by Carillo et al. (1998), FMDV viral protein 1 (VP1) from transgenic *A. thaliana* plants. Later on there was a lot of production of complete or fractionated VP1 with the aim of producing an effective, highly immunogenic, easy to use and cheap plant-made FMDV Ag vaccine. VP1 epitope 135 - 160 has been expressed in transgenic alfalfa (Widgorovitz et al., 1999; Dus Santos et al., 2002; Dus Santos and Widgorovitz, 2005), *A. thaliana* (Carillo et al., 1998; Dus Santos and Widgorovitz, 2005) and potato (Dus Santos and Widgorovitz, 2005), structural polyproteins- precursors for empty FMDV capsids, P1-3C and P1-2A3C have been expressed in transgenic alfalfa (Dus Santos and Widgorovitz, 2005; Dus Santos et al., 2005) and tomato (Pan et al., 2008), respectively, and two serotypes of the structural FMDV VP1 protein, O- and Asia 1-type have been expressed in transgenic forage maize (Zhang et al., 2011). When used as an experimental vaccine for either oral immunization by feeding fresh plant tissue or parental, intraperitoneal vaccination with transgenic plant extracts VP1 epitope induced FMDV-specific systemic immune response and gave protection against FMDV infection after experimental viral FMDV challenge (Carillo et al., 1998; Widgorovitz et al., 1999; Dus Santos et al., 2002), recombinant protein yields however were low, between 0.01 and 0.2 mg/g in transgenic alfalfa (Dus Santos and Widgorovitz, 2005), the highest level of expression of FMDV VP1 135-160 epitope expressed in alfalfa being 0.5 - 1 mg/g of TSP, which was 10

times higher than in the cases of Widgorovitz et al. (1999) and Carillo et al. (1998). No significant differences in transformation success, protein expression or immunogenicity were observed with regards of using different *A. tumefaciens* strains or host systems. Dus Santos and Wisgorovitz (2005) expressed FMDV VP1 P1-3C in transgenic alfalfa with expression level 0.01 - 0.2 mg/g of TSP when binary pRok plasmid and *A. tumefaciens* strain C58C1 were used and with expression levels of 0.005 - 0.01 mg/g of TSP in another case, in both cases the obtained recombinant peptide induced strong Ab and NA response as well as complete virological protection in vaccinated mice. Pan et al. (2008) expressed P1-2A3C FMDV VP1 polyprotein in tomato, its expression was lower than that obtained in FMDV-infected animal cells despite the use of KDEL, Kozak sequence and 35S promoter; however the immunogenicity in intramuscularly immunized guinea pigs was still very high, with Ab titres 21 days after the third vaccination 0.8 - 2 log₁₀. A FMDV-specific memory cell response was generated as well. When Zhang et al. (2011) expressed FMDV serotypes O- and Asia 1-type in transgenic forage maize, 3 - 5 transgene copies of VP1 fusion protein were found inserted in genomes of transformed plants and the transgene was found to be passed on to offspring. Ag induced protective systemic immune response when fed to animals.

2.4 Hepatitis B

Hepatitis B is one of the most frequent contagious diseases. It is caused by a hepatitis B virus (HBV) and mainly damages liver, can lead to liver cirrhosis or hepatocellular carcinoma. HBV is a retrovirus, one of the smallest eukaryotic DNA viruses (Guan et al., 2013). Negative HBV strand has 4 ORFs, *C*, *P*, *S* and *X* gene encoding four major viral proteins: core Ag (HBcAg/HBeAg), HBV DNA polymerase (HBV DNA P), surface Ag (HBsAg) and X Ag (HBxAg), respectively (Guan et al., 2013). Since the first reported plant-made HB Ag in *A. tumefaciens* strain LBA4404-transformed tobacco by Mason et al. (1992) there has been a huge research on expressing major HBV viral proteins. Recombinant HBsAg has been expressed in lupine and lettuce (Kapusta et al., 1999), carrot suspension cells (Zhao et al., 2002), soybean (Smith et al., 2002), peanut (Chen et al., 2002), tomato (Ma et al., 2002; Carolina and

Francisco, 2004; Wang and Li, 2008; Srinivas et al., 2008), cherry tomatillo (Gao et al., 2003; Guan et al., 2010), potato (Shulga et al., 2004), banana (Sunil-Kumar et al., 2005), apple core and leaves and tomato (Lou et al., 2005), NT-1 cells of tobacco (Sunil-Kumar et al., 2007) and lettuce (Kostrzak et al., 2011). Chen et al. (2002) reported a high level expression of HBsAg in peanut, obtained by *Agrobacterium*-mediated transformation, with the yield of 2.42 µg HBsAg/g of fresh weight. Oral immunization was shown to have triggered anti-HBsAg response both in mice (Kapusta et al., 1999; Gao et al., 2003) and humans (Kapusta et al., 1999; Thanavala et al., 2005). Many of HBsAg expression systems are still undergoing the process of development and optimisation and new plant species are being tested as possible expression systems. The most recent experiments include cucumber (Unni and Soniya, 2010), sandalwood embryogenic cell suspension cultures (Shekhawat et al., 2010), maize (Hayden et al., 2012; Hayden et al., 2015; Shah et al., 2015) and lettuce (Pniewski et al., 2011), as well as the most recent progress in using potato as host system (Rukavtsova et al., 2015). Even though the first effective parental hepatitis B vaccine by yeast has been developed more than 20 years ago, the vaccine still isn't accessible, effective or is cost-prohibitive for a lot of world's population, especially for areas that need it the most. A big problem is also the storage of the vaccine as the adjuvanted purified Ag protein loses its immunogenicity when frozen, thawed or stored for a week at 45 °C and is not thermostable (Shah et al., 2015). An oral, low-cost thermostable plant-made subunit vaccine could help overcome those problems. In 2011, Pniewski et al. expressed HBsAg in glufosinat-resistant transgenic lettuce to use as a prototype oral vaccine suitable for human immunization. Total HBsAg level in lyophilized tissue was 5 mg/g, whereas the mean content of VLP-structuralized Ag was 11 µg/g and in tablets 5 or 2.3 µg/g. Obtained vaccine consisted of 100 ng VLP-assembled Ag dosage, produced in transgenic lettuce and mice were orally immunized with lyophilized tissue without additional exogenous adjuvants. Two immunizations with 60-day interval in between them resulted in both mucosal and peripheral humoral immune response against HBV, with titres above the nominal protective titres (10 mIU/ml). When converted into tablets and stored at room temperature, HBsAg

content in lyophilized tissue was preserved for a year. Even though the lyophilisation reduced the HBsAg content for at least 90 %, the low dosage was still efficient. In 2010, Unni and Soniya expressed HBsAg in transgenic salad cucumber (*Cucumis sativus* cv. 'Swarnamukhi') plants as a part of trials to find new vaccine-producing plants suitable for raw human consumption. Using *Agrobacterium*-mediated transformation of *C. sativus* cotyledonary leaf section they produced transgenic *C. sativus* plants where HBsAg was successfully introduced and transcribed, with T-DNA being present as single copy in some of the plants and as multiple copy in others, the transgene insertion pattern that is typical for *Agrobacterium*-mediated transformation. Soluble proteins extracted from transformed plants showed a distinct band with molecular weight expected for the HBsAg gene. Protein expression levels were low, 0.006 % of TSP (11.5-50 ng HBsAg/mg TSP), which was attributed to the absence of signal peptides for increasing protein accumulation, such as ER SEKDEL.

Besides using different plants, Shekhawat et al. (2010) expressed HBsAg in embryogenic cells suspension cultures of sandalwood (*Santalum album*). The team used *Agrobacterium*-mediated transformation technique, a C-terminal ER retention signal was added to hepatitis HBsAg gene and eight different growth medium additives were tested with the aim of improving the HBsAg expression in transgenic cells. Maximum HBsAg expression in untreated transformed cells was 11.09 µg/g of fresh mass, while cells treated with 30 mM trehalose reached 19.95 µg/g, addition of 0.4 % DMSO increased the expression by 1.36-fold, addition of gibberellic acid (1.45 µM) by 1.48-fold, doubled concentration of CaCl₂ in basic MS medium by 1.28-fold and 0.001 % Tween-20 by 1.34-fold. The biggest increase was obtained by addition of 54.88 mM mannitol, with the increase of 1.7-fold. Due to bigger genetic stability of sandalwood as opposed to previously investigated soybean suspension cells (Smith et al., 2002) this study proved promising for further investigations of stable HBsAg expression in suspension cells of sandalwood.

The first report on successful expression and immunogenicity of bioencapsulated HBsAg was done in 2012 by Hayden et al., in maize, as the first

commercially feasible oral subunit vaccine production for a major disease. HBsAg expression was driven by either polyubiquitin or the embryo-preferred globulin 1 promoter and the effect of different signal sequences on the HBsAg accumulation in grains was tested. Accumulation of the HBsAg was the highest when driven by embryo-preferred globulin 1 promoter, with the highest yielding seed accumulating over 0.25 % TSP. Germ enrichment and oil extraction from the seeds of plants produced by backcrossing of the 2 of the highest-yielding plants resulted in the total of 166 µg HBsAg/g of dry maize material, 20-fold higher than in the highest previously reported case. Orally immunized mice elicited both strong mucosal and systemic immune response. As the next step towards maize-produced oral HBV vaccine, Hayden et al. (2015) recently carried out a study to determine whether or not oral delivery of maize-produced HBsAg doses establish long-term immunologic memory in mice over a one year time period. Using the plant material from the previous study, Hayden et al. (2015) produced hybrid HBsAg seeds which were then processed into supercritical fluid CO₂ extraction (SFE)-defatted wafers. Serum IgG, serum IgA and fecal IgA responses were detected in mice intramuscularly immunized with commercial HBV vaccine Recombivax and fed with SFE-defatted wafers as well as in mice immunized with Recombivax and immunized with it afterwards. Storage and processing of wafers resulted in the final wafers containing 567 µg HBsAg/g, 3-fold more than in the beginning. Total serum IgG (mIU/ml) showed clear evidence of immunologic memory over one year of oral and parenteral administration, geometric mean titres (mIU/ml) were high both in the case of HBsAg- and Recombivax-treated mice. Obtained HBsAg levels in seeds and resulting wafers are the highest reported Ag concentrations in plants to date. In the same year, the biochemical and biophysical characteristics of SFE-defatted maize-derived HBsAg were evaluated (Shah et al., 2015). Expression of HBsAg in plants has been recently enhanced by Hayden et al (2014) by fusing HBsAg to barley alpha amylase signal sequence (BAAS) and preferentially expressing it in the germ fraction of the hybrid maize seed, which resulted in HBsAg expression at a level of 1023 µg/g, the highest reported HBsAg in plants up-to-date. Recently, Rukavtsova et al. (2015) tested the immunogenicity of marker-free

transgenic potato-produced HBsAg in mice. The team developed a novel way of constructing a transgenic marker-free plant, inserting the HBsAg (driven by 35S CaMV promoter) into the marker-free pBM vector and performed *Agrobacterium*-mediated transformation. HBsAg level in the tubers of selected transgenic potato lines was up to 1 µg/g of wet weight. Existence of HBsAg multimers was confirmed by gel filtration. Anti-HBsAg levels in mice fed with transgenic potato tubers began increasing on day 36-50 after the 1st feeding and by day 70 the Ab content was up to 170 mIU/ml. After being experimentally challenged with recombinant yeast hepatitis B vaccine, that content rose up to 350 mIU/ml within 15-34 days after infection in orally immunized mice, staying high above the minimum protection level even 120 days after the start of the experiment (up to 100 mIU/ml). When having been given additional 3 doses of HBsAg (320 days after the start of the experiment), the HBsAg Ab levels in serum of previously immunized mice increased up to 185 mIU/ml, with animals preserving stable immunological memory of the infection. This was the first experiment using transgenic plants as a source of hepatitis B edible vaccine that lasted longer than 24-38 weeks, the way that was normal in similar experiments of other authors.

2.5 Rabies

Rabies is a globally widespread infectious disease affecting central nervous system in both animals and humans. It is caused by the Rabies virus. Despite the long research history it still remains incurable while vaccination is used as a mean of its prevention. Vaccines currently available on the market are obtained by propagating fixed Rabies virus strains in various animal cell cultures or chicken embryos (Starodubova et al., 2015). Despite being safe and efficacious, vaccines produced in such way can greatly vary between different producers, their efficiency can be significantly reduced by storage and transportation, are very costly, can sometimes cause adverse side effects and require 3 - 5 doses to provide sufficient immune protection (Rosales-Mendoza, 2015). In the last 20 years there has been a lot of research and progress in developing the fourth generation of rabies vaccines based on rabies glycoprotein (G protein) (Starodubova et al., 2015). Neutralizing Abs against G protein have been shown to be

capable of preventing rabies infection. The pioneering work in expressing G protein in transgenic plants was done in 1995 by McGarvey et al. by expressing an unmodified G protein gene under the control of the CaMV 35S promoter in *Agrobacterium*-transformed tomato leaves and fruits. Since then, G protein has been expressed on its own in plants such as tobacco (Modelska et al., 1997; Yusibov et al., 1997; Ashraf et al., 2005), spinach (Modelska et al., 1997), carrot (Anaya et al., 2009) and maize (Loza-Rubio et al., 2012). A full-length rabies virus nucleoprotein has also been expressed in tomato (Perea Arango et al., 2008), cholera toxin subunit B (CT-B)-rabies glycoprotein fusion protein has been expressed in tobacco (Roy et al., 2010) and tobacco seeds (Tiwari et al., 2009), chimeric peptide containing rabies G protein and N protein determinants was expressed in tobacco and spinach (Yusibov et al., 2002) and a rabies glycoprotein- ricin toxin B chain fusion protein has been expressed in tomato hairy roots (Sigh et al., 2015). A similar expression approach that was used by Ashraf et al. (2005) to express a synthetic, codon-optimised G protein gene in tobacco for the first time. Native signal peptide was replaced by *N. tabacum* pathogenesis-related protein PR-S and an ER KDEL signal sequence was added to the C-terminus of the G protein gene. Selected nuclearly transformed plants expressed G protein at 0.38 % TSP in leaves. Intraperitoneal immunization of mice resulted in protective immunity against intracerebral challenge with live rabies virus. In 2012, Loza-Rubio et al. expressed G protein in maize seeds and evaluated its immunogenicity in polygastric species for the first time. As a transformation method biolistics was used. The average expression level in obtained transgenic plants was 25 µg/g of fresh tissue, equalizing about 1.4 % TSP, which is the same amount as was obtained in carrot by Anaya et al. (2009). When orally immunized with 2 mg of obtained G protein one single time, sheep (the main victims of rabies infections in Latin America) elicited anti-rabies neutralizing serum Ab and a protective immunity against rabies virus infection with a survival rate of 83 % was obtained, the same as the efficiency obtained by inactivated commercial vaccine (Loza-Rubio et al., 2012). Apart from G protein, a full-length rabies virus nucleoprotein (N protein) was transiently expressed in *Agrobacterium*-transformed tomato and *N. benthamiana* plants in 2008 for the first

time (Perea Arango et al., 2008). N protein was expressed at levels ranging from 0.1 - 0.5 mg/g to 1 - 4 % TSP in tomato fruit and up to 4 mg/g and 45 % TSP in *N. benthamiana* leaves and it was found to have induced Ab production in both orally and intraperitoneally immunized mice, whereas only 50 % of the latter were protected against a peripheral viral challenge. Yusibov et al. (2002) expressed G and N protein in the form of a chimeric peptide together with tobacco virus mosaic proteins in tobacco and spinach. Parenterally immunized mice showed immunity to rabies virus and an anti-rabies immune response was elicited in human volunteers orally immunized by transgenic chimeric peptide from spinach. With the aim of making it more effective, rabies virus proteins have been expressed as fusion proteins with well-known potent mucosal adjuvants. Rabies-CT-B fusion protein has been expressed in tobacco (Tiwari et al., 2009; Roy et al., 2010). In the case of Roy et al. (2010) accumulation level was 0.4 % TSP, fusion protein formed pentameric protein which was biologically active in binding to the GM1ganglioside receptor. However its *in vivo* activity and immunogenicity have not been tested. Recently, Singh et al. (2015) expressed rabies virus glycoprotein fused to ricin toxin B chain in tomato hairy roots. Fusion gene construct contained N-terminal ER-targeting Calreticulin signal sequence from tobacco and C-terminal ER-retention signal SEKDEL and was under control of CaMV 35S double enhancer promoter. Its expression was between 1.4 and 8 µg/g of tissue, with the highest yield being 1.14 % TSP. The highest yielding tomato line, containing single copy of the transgene stably integrated into nuclear genome, was used in bench-top bioreactor for optimization of scale-up process. By optimizing the cultivation parameters over the span of 3 weeks, the team managed to increase the growth rate in the bioreactor 49.3-fold. Partially purified fusion protein was capable of inducing immune response in intra-mucosally immunized mice (Th2 lymphocyte immune response) but due to the low titres of Abs protein quantity should be further optimized in order to obtain better responses both after the primal and booster dose. The use of hairy roots for production of vaccine antigens offers several advantages to their expression in whole plants: the ability to generate plants and store their germplasm long-term, the absence of toxic

compounds such as alkaloids, obtaining large quantities of protein in short time etc.

2.6 Rotavirus

Rotaviruses are the main cause of gastroenteritis in children and animal offspring, causing severe annual economic losses (especially) in cattle; over 500 000 annual deaths are estimated in humans, mainly in children living in developing countries in South Asia and sub-Saharan Africa due to insufficient hygienic conditions. Triple-layered rotavirus virions contain a genome of 11 dsRNA encoding 6 structural and 6 non-structural proteins. The capsid has 3 layers, named VP2, VP6 and VP7 (the outermost) with VP4 protein forming spikes on its surface. In the host intestine VP4 gets cleaved to VP5 and VP8* (Pera et al., 2015), especially the latter being highly immunogenic and playing a major role in rotavirus infectivity. The outer capsid layer, VP7, also contains several epitopes, important for viral activity. Therefore, VP7 and VP4 and its derivatives VP8* and VP5 were chosen as main candidate antigens for subunit vaccine development (Bergeron-Sandoval et al., 2011). Rotaviruses are divided into 7 groups (A-G). Group A contains 4 sub-groups based on the antigenic properties of VP6. Currently there are around 140 different genotypes of the human rotavirus described (genotype G, P or G/P). For human rotavirus there are currently two live vaccines available, monovalent vaccine based on the most common serotype G1P[8] and pentavalent G1-G4 P[8] vaccine. Due to their expensiveness making them unavailable to the majority of the population in the developing world and the recent emergence of several novel serotypes there is however a big demand for developing new and better rotavirus vaccines (Pera et al., 2015), an aim for which also transgenic plants have been utilized a lot during the recent years. Rotavirus capsid proteins have been expressed by various research groups: VP6 has been expressed in tomato (Chung et al., 2000; Chung et al., 2001), alfalfa (Dong et al., 2005), potato (Yu and Langridge, 2003), VP7 has been expressed in potato (Wu et al., 2003; Choi et al., 2005), VP8* in transplastic tobacco plants (Lentz et al., 2011), bovine VP8* in potato (Matsumura et al., 2002) and *N. benthamiana* in recombinant form (Perez-Filgueira et al., 2004a). In addition, NSP4-CT-B fusion protein has been

expressed in potato (Arakawa et al., 2001; Kim and Langridge, 2004), VP6-BSSV fusion protein in *Chenopodium amaranticolor* (Zhou et al., 2010) and VP2, VP6 and VP7 have been co-expressed in the form of VLPs in tobacco (Yang et al., 2011). These experiments proved that rotavirus coat proteins can be successfully expressed in various plant tissues where they accumulate to relatively high levels, as high as 0.28 % TSP of VP6 in tobacco (Dong et al., 2005) and 1.5 % TSP of rotavirus VLPs in tobacco (Yang et al., 2011). Obtained proteins were also capable of inducing both mucosal and systemic immune response in orally immunized mice as well as provide passive immunity against rotavirus challenge in suckling mice born to immunized mother: 60 % of pups in the case of VP6-BSSV from *C. amaranticolor* (Zhou et al., 2010), 85 % of pups in case of bovine VP8* from transplastic tobacco (Lentz et al., 2011) and bovine VP8* from tobacco (Perez-Filgueira et al., 2004a). The most recent research includes transient expression of a single antigen and dimeric combinations of human VP7 and truncated VP4 fused with fljB flagelling subunit from *Salmonella typhimurium* in *N. benthamiana* (Bergeron-Sandoval et al., 2011) and a vaccine candidate against the newly emerged G9P[6] strain, produced by transient expression in *N. benthamiana* (Pera et al., 2015). Bergeron-Sandoval et al. (2011) expressed codon-optimized full-length VP7, truncated VP4 and dimeric combinations of VP7, VP4 and fljB flagellin fused to 5'UTR of tobacco etch virus which works as a transcription enhancer, in leaves of *N. benthamiana* with relatively high yields: 5 µg/g for truncated VP4, 4 µg/g for flagellin, 31.97 µg/g for VP7-flagellin dimer and 12.3 µg/g for VP4-flagellin dimer. In the case of VP7 and VP7-VP4 dimer there was mild leaf necrosis whereas all the other proteins were stably expressed. The amount of obtained VP4 was fit to the amount of VP8* previously obtained in *N. benthamiana* plants when TMV-based vector was used for transformation (Perez-Filgueira et al., 2004a). Expression of VP7 however was much lower than that previously reported in transgenic potato tubers (40 µg/g) (Li et al., 2006), probably due to differences between the plant systems used, but the system tested in this report provides much faster and efficient production of antigenic proteins than systems using stable transformation. As can be seen, it is possible that fusion of VP proteins with

flagellin results in much higher protein expression, which could be used in production of rotavirus vaccines. Mice, subcutaneously immunized by VP4 and VP7, generated weak immune responses against rotavirus while mice immunized by dimeric fusion proteins generated strong, anti-flagellin immune response. As flagellin is known to have protected mice against chemical, bacterial, viral and radiation challenge without adverse side effects and induces many different cytoprotectant activities, Bergeron-Sandoval et al. (2011) propose it as a promising candidate for vaccine adjuvant. Pera et al. (2015) in term successfully expressed VP2 and VP6 in the form of VLPs in *N. benthamiana* by transient *Agrobacterium*-mediated transformation. Because the expression of VP7 and VP4 was not successful, the team tried to produce more appropriately immunogenic particles and created 3 fusion proteins, adding VP8* to the VP6 and co-expressing it with VP2. The team tested the effect of different ODs, intracellular targeting and tomato spotted wilt virus silencing suppressor protein (NSs) on protein expression and yield; they also used the tomato spotted wilt virus suppressor protein in order to optimise the expression. VP6 was successfully expressed in cytoplasm (where the addition of NSs enhanced the accumulation), chloroplasts (where the NSs addition reduced the accumulation), and apoplast and ER (where the NSs addition increased the accumulation on day 3 out of 7, the day on which the expression was at the maximal level). The addition of sequences, targeting the VP6 to intracellular compartments, did not significantly increase its accumulation. VP2 was expressed in cytoplasm, ER, apoplast and chloroplasts only in addition of NSs, at significantly lower average level than VP6. VP7 and VP4 have not been successfully expressed at all. The expression of VP8*/VP6 was only successful when binary plant vector pEAQ-HT was used, but not when plasmids from pTRA family were used. The highly immunogenic epitope of VP8* was fused to either N- or C-terminal end of VP6 and successfully expressed in cytoplasm, whereas VP6/8*C was expressed faster and at higher levels than VP6/8*N. VP2 was co-expressed with all the proteins, expressed in cytoplasm, and was found to be expressed at the highest level when co-expressed with VP6 (Pera et al., 2015). The fusion proteins did not form VLPs but based on previous studies by other authors the team suspects they could still retain their high

immunogenicity, which would have to be evaluated in animal experiments.

2.7 Newcastle disease virus

Newcastle disease virus (NDV) is the cause of an economically important avian diseases, heavily affecting global poultry industry. The main candidates for developing a vaccine against the disease are fusion protein (F) and hemagglutinating-neuraminidase protein (HN) since they are both exposed on the surface of the virus and take part in the infection. HN protein has been expressed in *N. benthamiana* (Gomez et al., 2009; Lai et al., 2013), potato (Berinstein et al., 2005) and tobacco (Hahn et al., 2007), F protein has been expressed in transgenic rice (Yang et al., 2007), potato (Berinstein et al., 2005) and maize (Guerrero-Andrade et al., 2006). These experiments proved that HN and F proteins can be successfully expressed in transgenic plants and that obtained proteins are immunogenic when delivered to either mice or chickens. Both maize-produced NDV F protein (Guerrero-Andrade et al., 2006) and tobacco-produced HN protein (Hahn et al., 2007) have been found to be immunogenic in orally immunized chickens; oral immunization with F protein gave 100 % protection of chickens against nasal challenge with NDV, while in the case of HN protein protection against nasal NDV challenge failed but the authors reported a big increase in anti-HN serum IgG levels 28 days after vaccination, increased by 2-fold in 40 % of examined chickens and 4-fold in 20 % of examined chickens (Hahn et al., 2007). HN and F proteins, co-expressed in potato, have been shown to elicit anti-NDV antibodies in intraperitoneally immunized mice and serum IgG at levels comparable to those elicited in mice fed with non-transformed plants soaked in NDV (Berinstein et al., 2005), while the intestinal fluids of those mice also showed considerable IgA and IgG levels. F protein, stably expressed under ubiquitin promoter or rice glutelin promoter in rice, elicited anti-NDV serum IgG in intraperitoneally immunized mice (Yang et al., 2007). From the point of view of expression level for each of the expressed protein it was noted that F protein was expressed higher in maize kernels than in rice (0.9 - 3 % TSP and 0.0025 - 0.0055 % TSP, respectively) (Guerrero-Andrade et al., 2006; Yang et al., 2007), while the expression of HN protein was higher when co-expressed with F protein in potato than when

expressed on its own in tobacco (0.3 - 0.6 $\mu\text{g/g}$ total leaf protein and 0.069 % TSP, respectively) (Berinstein et al., 2005; Hahn et al., 2007). When transiently expressed in *N. benthamiana* as a pSPHnHN/KDEL, under the control of rubisco small subunit promoter, fused to its own KDEL ER retention signal, recombinant pSPHnHN/KDEL protein accumulated to the highest levels from all of the 5 tested constructs (Gomez et al., 2009). When potato was transformed with pSPHnHN/KDEL as described by Berinstein et al. (2005), the protein expression was as high as 3 $\mu\text{g/g}$ of total leaf protein, which is 10-fold higher than levels obtained by expression of HN under the control of CaMV 35S promoter in potato (Berinstein et al., 2005). In 2006, US Department of Agriculture Center for Veterinary Biologics gave regulatory approval and a license to Dow Agro Sciences LLC for an injectable vaccine against NDV based on HN protein produced in tobacco suspension cell line. In a proof-of-concept study, two subsequent immunizations gave 90 % protection of birds against a lethal virus challenge (Gomez-Lim, 2014). This vaccine is the first fully-licensed plant-cell-produced vaccine for animals in the United States and the first plant-made product to be licensed by USDA's Animal and Plant Health Inspection Service (USDA ..., 2006). The company has, however, shown no interest in commercialization of the vaccine up to date. With the aim of improving the HN expression in plant host system, Lai et al. (2013) recently expressed functional ectodomain of HN protein (eHN) from NDV strain AF2240 in *N. tabacum* BY-2 cells, which had been proven suitable for production of various recombinant proteins. The eHN translation was enhanced by 5'-UTR region of *N. tabacum* alcohol dehydrogenase gene driven by CaMV 35S promoter. An eHN cDNA was successfully integrated into plant cell genome and actively transcribed. For all 8 transformants showing the highest levels of eHN mRNA transcripts, immunoblot detected a protein band the size of 66 kDa, corresponding to the predicted eHN molecular weight. The eHN protein expression was stable and accounted up to 0.2 - 0.4 % TSP, significantly more than in previous studies (Hahn et al., 2009; Gomez et al., 2009) using strong constitutive promoter and translation enhancer sequence. Localization of eHN-GFP in BY-2 protoplasts showed predominant accumulation of the protein in cytosol. Mice, intraperitoneally

immunized with 5 μg of purified plant-produced eHN in incomplete Freund adjuvant 4 times, elicited anti-NDV antibodies, immune response enhancing with each additional boost and reaching the peak by day 67 (Lai et al., 2013). Similar responses were obtained in mice immunized with full-length potato- and tobacco-produced HN (Berinstein et al., 2005; Hahn et al., 2007, respectively). These results proved that truncated HN protein, lacking the transmembrane domain, still maintains its biological and functional activity in murine model and is capable of inducing an immune response closely resembling that of the native protein, so it can serve as a promising candidate antigen for development of a NDV subunit plant-made vaccine.

2.8 Norwalk virus capsid protein

Norwalk virus is the cause of epidemic acute gastroenteritis in humans both in developed and developing countries. It spreads via water, food and human contact. 42 % of acute epidemic gastroenteritis in the United States is estimated to be caused by Norwalk virus (Tang and Page, 2013). Currently there is no Norwalk virus vaccine available. The main candidate antigen for its development is, however, Norwalk virus capsid protein (NVCP), which has been expressed in the form of virus-like particles (VLPs) in potato and tobacco (Mason et al., 1996), potato tubers (Tacket et al., 2000), tomato fruit (Huang et al., 2005), *Nicotiana benthamiana* (Santi et al., 2008; Huang et al., 2009; Souza et al., 2013) and lettuce (Lai et al., 2012). Immunogenicity of produced VLPs has been evaluated in mice, fed by gavage (Mason et al., 1996), human volunteers (Tacket et al., 2000) and orally immunized mice (Huang et al., 2005; Santi et al., 2008). Evaluated VLPs have been found capable of inducing sufficient antigen-specific systemic and mucosal immune response, even in the case when freeze-dried transgenic NVCP tomato fruit was used (Huang et al., 2005). Protein accumulation in transgenic tomato was much higher than in potato tubers, 26.7 $\mu\text{g/g}$ in tomato fruit and 1.4 -3.6 $\mu\text{g/g}$ in potato tubers (Huang et al., 2005; Tacket et al., 2000, respectively). The best expression of the NVCP was obtained when the first two Met residues of the N-terminus had been eliminated from the native NVCP (Huang et al., 2005). Santi et al. (2008) developed an efficient tobacco mosaic

virus- derived transient expression system and successfully expressed NVCP in leaves of *N. benthamiana* at levels 0.8 mg/g 12 days post-infection as a more rapid alternative to expressing NVCP in plants and found it to be immunogenic in orally immunized mice. The procedure of expressing NVCP VLPs has been further improved by Huang et al. (2009) who optimized geminivirus-derived DNA replicon vectors for expression of hepatitis B core antigen and NVCP in *N. benthamiana* leaves. Using bean yellow dwarf virus-derived vector and Rep/RepA-supplying vector for agroinfiltration of leaves and co-expressing the tomato bush stunt virus P19 protein as a mRNA stabilizer the team was able to greatly enhance the transgene expression and shorten the time needed for the final product, thereby producing as much as 0.34 mg/g NVCP VLPs in 5 days' time. The same system was tested in lettuce by Lai et al. (2012). NDCP was found correctly expressed in lettuce leaves, with the highest accumulation at 4 days post-infection and an average expression level of 0.2 mg/g. This is comparable to the results of Huang et al. (2009) and is the highest expression level of any non-chloroplast-derived vaccine component ever reported in lettuce plants (Lai et al., 2012). Recently, Souza et al. (2013) tried to optimize expression of norovirus-like particles, such as NVCP-VLPs, in plants, utilizing a binary vector and co-expression of PTGS suppressor to increase the target protein yield. In the series of experiments in *Agrobacterium*-transformed *N. benthamiana* leaves, the team first examined the effect of 4 post-transcriptional gene silencing (PTGS) suppressors on protein expression with GFP as a reporter in *N. benthamiana* co-infiltrated with PTGS and GFP, recording the observed amount of GFP. The most effective PTGS, 126 kDa Pepper mild virus protein, was then used for co-expression with major and minor capsid genes of NVCP (vp1 and vp2, respectively) with a 3'UTR. Obtained VLPs were purified by sucrose gradient centrifugation. While the previous studies all used so-called magnICON viral vectors and transient expression systems, this is the first trial of expressing inserts, longer than just vp1 in plant expression systems. Even though the team used *N. benthamiana* -optimized codons, the expression level remained the same as that of vp1 isolated from the virus itself. Tang and Page (2013) adapted the glucocorticoid-inducible expression

system developed by Aoyama and Chua (1997) and Ouwerkerk et al. (2001), to express NVCP in *Agrobacterium*-transformed cell suspension cultures of tobacco, rice, slash pine and cotton, using dexamethasone (DEX) as an expression inducer. Transformation vector contained hygromycin phosphotransferase, chimeric transcription factor GVG and NVCP gene. For each plant species, the effect of different dexamethasone concentrations (1.25, 2.5, 5, 10, 20 and 40 mg/l) on transgene expression have been tested. For all concentrations there was clear transgene expression 2 days after the addition of the inducer to the medium, and no expression of the transgene was found in plants with no inducer added. Maximum NVCP expression was obtained at 10 mg/g DEX; greater concentrations were deleterious to transgene expression.

2.9 HIV

Human immunodeficiency virus type 1 (HIV-1) is the cause of AIDS (acquired immunodeficiency syndrome), a deadly infectious global disease, affecting more than 34 million people worldwide (Lotter-Stark et al., 2012). The great genetic variety among HIV-1 subtypes, high mutation rate and biological properties of HIV-1 regulatory proteins by avoiding immune response (Cueno et al., 2010) significantly worsen the process of developing an efficient vaccine. Various HIV proteins have been expressed in plant systems so far: HIV-1 p24 in tobacco (Zhang et al., 2002; Perez-Filgueira et al., 2004b) and *A. thaliana* (Lindh et al., 2014), HIV 1 P55 Gag polyprotein in tobacco chloroplasts (Scotti et al., 2009) and tobacco plants both in full length and truncated form (Meyer et al., 2008), HIV-1 Tat in potato (Kim and Langridge, 2004a) and tomato (Cueno et al., 2010), HIV-1 Nef in tobacco (Marusic et al., 2007) and *N. benthamiana* (Lombardi et al., 2009; Circelli et al., 2010), HIV-1 CA capsid protein VLPs in *Lycium barbarum* (Du et al., 2004), HIV-1/HBV fusion protein has been expressed in tobacco (Greco et al., 2008; Guetard et al., 2008) and *A. thaliana* (Greco et al., 2008). Tat regulatory element of the simian-HIV-1 virus (SHIV 89.6 Tat) has also been expressed in potato (Kim and Langridge, 2004b), fused to cholera toxin subunit B. As an important early marker of HIV-1 infection p24 protein, a major component of the gag polyprotein, is a promising candidate for developing an oral vaccine used to offer immediate

protection of the gut-associated lymphoid tissue (GALT) against HIV-1 challenge. The first researches in expressing p24 in plants were done by Zhang et al. (2002), who stably expressed p24 gene cassette in *Agrobacterium*-transformed tobacco with the average yield 3.5 mg/g leaf soluble protein and was proven antigenic by Western blot. Perez-Fligueira et al. (2004b) modified tobacco mosaic virus TMV-30B to express HIV-1 p24 in tobacco. The addition of 7 His residues to the C-terminal end of the transgene was used to transiently express HIV-1 p24 in tobacco leaves with yields as high as 2.5 mg of purified p24/25 g of leaf tissue, which would suffice for 20000 routine diagnostic tests for HIV-1 as carried out in accordance with the WHO standards. Rabbits, immunized with purified p24 diluted in buffer in the presence of Freund adjuvant, developed a specific anti-p24 humoral response following the second immunization and p24 was also proven functional when used for Western blot assay to confirm HIV-1 test results in a set of Zambian patients. So despite the denaturing conditions, IMAC columns, used to purify p24 expressed by TMV-p24-HISc modified vector, the protein retained its function and efficiency. In 2011, Gonzales-Rabade et al. reported a successful expression of HIV-1 p24 and HIV-1 p24-Nef fusion protein in the chloroplasts of transplastomic tobacco. Expression levels obtained in leaves were 4 - 40 % TSP. Oral immunization of mice by gavage, with CT-B as an adjuvant (ratio p24:CT-B 1:1, i.e. 10 µg of each), elicited strong p24-specific serum IgG responses, with IgG sub-classes found in sera of immunized mice after subcutaneous immunization indicating activation of humoral immune response as well. Recently, Lindh et al. (2014) carried out a series of experiments, using previously obtained transgenic carrots, expressing 90 ng/g FW, and high- and low-yielding *A. thaliana* (expressing 366 ng/g and 34 or 17 ng/g FW, respectively) were used for oral immunization of mice. Both plants were used in either fresh or freeze-dried form. 6/7 experiments contained fresh plant tissue and 1 freeze-dried, mice in 4/7 experiments were allowed to feed freely and 3 controlled by tube feeding. The effect of the plant tissue for all experiments was evaluated by ELISA, two weeks after the p24 intramuscular boost. In the case of carrot, high amounts of p24-specific IgG were found in sera of immunized mice 2 weeks after the boost.

Immunization with freeze-dried *A. thaliana* by gastric tube did not result in detectable p24-specific Abs while immunization with fresh tissue gave results similar to those in the case of transgenic carrot. Interestingly, mice immunized with low-level p24 extract had higher response to the antigen boost while mice immunized with high-level p24 extract had much weaker immune response. When CT-B was added to the antigen, there was no serum immune response whatsoever. In the case of free feeding, anti-p24 IgG were observed in the serum of mice fed with low-level p24 *A. thaliana* after the p24 boost, while no anti-p24 IgG were detected in the serum of mice fed high-level p24 *A. thaliana*. This was the first dose-dependent HIV-1 p24 antigen delivery study using transgenic *Arabidopsis* (Lindh et al., 2014). Immunizations with fresh transgenic carrots (non-mandatory eating, free feeding, 720 ng p24/dose) induced anti-p24 IgG response in the serum, following the p24 boost, however the observed IgG levels were low. Fresh *A. thaliana* free feeding induced weak IgG response no matter which dose was delivered (28, 200, 620 ng or 2.2 µg p24/dose). Low-level p24 extract fed by tube (20 ng/dose) was more efficient than high-level extract (460 ng/dose), results supporting the threshold value of 20 ng of antigen as a minimum to induce detectable immune response, as described by Beyer et al. in 2007.

2.10 Avian influenza virus H5N1

Highly pathogenic avian influenza is a deadly disease that had initially been affecting only poultry, thus causing great economic losses, but has also been representing a serious risk for human health since the first outbreak of highly pathogenic influenza (H5N1) in humans in Hong Kong in 1997 (Guo et al., 2012) in Asian countries as well as in Europe and Africa. Development of safe, cost-effective and efficacious vaccines is thus needed to reduce the economic impact of the disease on agriculture and prevent possible future pandemic outbreaks. The cause of the disease is highly pathogenic influenza A virus subtype H5N1. The main targets for subunit vaccine development have been its principal surface antigen hemagglutinin (HA), which plays a major role in functional activity of the virus and is also a main target for neutralizing antibodies of the host, and the M2e viral peptide, the extracellular domain

of the viral protein M2, which is highly conserved between various influenza strains and therefore a promising candidate for a universal influenza vaccine. H5N1 HA has been expressed in *N. benthamiana* in full-length (Kalthoff et al., 2010) and in the form of VLPs (Landry et al., 2010), in *Lotus corniculatus* (Guo et al., 2012) and most recently, duckweed (Bertran et al., 2015). In addition, recombinant HA from A/Bar-headed Goose/Qinghai/1A/05 (clade 2.2) and A/Anhui/1/2005 (clade 2.3) have been expressed in *N. benthamiana* (Shoji et al., 2009). Kalthoff et al. (2010) were the first to evaluate the immunogenicity of plant-produced full-length HA in chicken. Using different magnICON vectors, HA was targeted either to cytosol, apoplast or chloroplasts and the apoplast-targeted HA was expressed at the highest level (0.3 g/kg of fresh leaf biomass), especially when tobacco calreticulin signal peptide was used. Chickens, intramuscularly immunized with vaccine containing HA with copolymer, catatonic lipid-DNA complex or Freund adjuvant, elicited strong neutralizing Ab responses against H5N1 and were protected against the lethal virus challenge. Landry et al. (2010) reported the ability of alum-adsorbed H5N1 HA VLPs to induce cross-reactive anti-H5N1 antibodies in ferrets as well as good tolerance and immunogenicity of the experimental alum-adsorbed vaccine in adult human volunteers in Phase I clinical trial. Immunization with HA did not trigger an immune response to plant-specific carbohydrate determinants neither were the known allergies to plant derivatives or presence of detectable anti-plant-specific glycans IgG levels lead to immune response against N-glycans present on the HA used in vaccine (Kalthoff et al., 2010). Recently, this finding was supported by the results of the research carried out by Ward et al. (2014), who evaluated the immune and possible allergic response to *N. benthamiana*-produced VLPs carrying HA from H5 or H1 influenza viruses, in 280/349 humans. Subjects were intramuscularly immunized with 1 (H1) or 2 (H5) doses of 5-45 µg HA/dose and monitored for 6 months. 34 % of them developed transient IgG and in some cases IgE against plant glyco-epitops but no hypersensitivity or allergy was observed. Guo et al. (2012) were the first to successfully express biologically functional H5N1 HA in *L. corniculatus*, the gene expression being driven by CaMV 35S promoter, using *Agrobacterium*-

mediated transformation. Transformation frequency in successfully transformed cotyledon fragments was 58.8 % as determined by PCR or Southern blot analysis, while transformation of hypocotyl gave no results. Using pBI-MARS-HA plasmid resulted in HA expression with the maximum of 0.00786 % TSP, much higher than in the case of pBI-HA plasmid (0.00408 % TSP), which, considering the use of *L. corniculatus*-optimized codons, MARS and CaMV 35S promoter, is still very low. Recently, synthetic HA gene from H5N1 virus A/chicken/Indonesia/7/2003 (Indo/03) was expressed in duckweed. Its efficacy was first tested in birds immunized with 0.2 µg or 2.3 µg HA and challenged with 10⁶ mean chicken embryo infectious doses (EID₅₀) of homologous virus strain (Bertran et al., 2015) and in birds immunized with 0.9 µg or 2.2 µg HA challenged with 10⁶ EID₅₀ of heterologous H5N1 virus strains A/chicken/Vietnam/NCVD-421/2010 (VN/10) or A/chicken/West Java/PWT-WIJ/2006 (PWT/06) (Bertran et al., 2015). Almost all birds immunized with 0.2 or 2.3 µg of HA elicited anti-Indo/03 antibodies and were protected against homologous virus challenge, 100 % of birds immunized with either dosage of HA showed protection against VN/10 challenge while birds challenged with PWT/06 showed 50 % mortality when immunized with 0.9 µg HA and 30 % mortality when immunized with 2.2 µg HA. Only birds challenged with VN/10 developed humoral immune response against the challenge antigen. Shoji et al. (2009) expressed 2 strains of H5N1 in *N. benthamiana*. Sequences for each gene were optimized for plant expression using tobacco PR-1 signal peptide at the N-terminus and ER retention signal KDEL and poly-histidine tag at C-terminus and *Agrobacterium*-mediated transformation was performed. Subcutaneous immunization with purified antigen from both strains elicited hemagglutinin inhibition (HI) and virus neutralizing (VN) antibodies in mice, with HI titers > 1:40, which is the minimal titer consistent with protective immunity in humans. M2e has been expressed in tobacco (Tarasenko et al., 2013) and recently, in duckweed (Firsov et al., 2015) and also in a form of a hybrid protein M2eHBC, fused to hepatitis B core antigen (Ravin et al., 2012). Tarasenko et al. (2013) expressed synthetic, codon-optimized 22-, 30- or 43-amino acids long terminal fragments of the M2 protein, encoding 3 different variants of M2e peptide (M122, M130 and M143,

respectively, with M122 and M130 encoding truncated M2 protein variants), fused with N-terminus of β -glucuronidase, in *Agrobacterium*-transformed *N. tabacum*. Transformation by vectors containing M122 or M130 resulted in successful expression of both variants of M2e peptide, in the form of M122- β -glucuronidase and M130- β -glucuronidase fusion proteins. In plants transformed with vector containing M143 gene for non-truncated M2e, the protein was observed only in plants grown *in vitro* and disappeared when the plants were transported into greenhouse. M2eHBC hybrid protein has been transiently expressed in *N. benthamiana* using a recombinant potato X virus-based viral vector. M2eHBc accumulated in leaf tissue at amount 1 - 2 % TSP in the form of VLPs. Immunogenicity of the VLPs was evaluated in intraperitoneally immunized mice. Three-fold immunization induced high titers of serum IgG targeting both the synthetic polypeptide, used in immunization (M2eHBc polypeptide G19) and its corresponding peptide sequence from heterologous influenza virus strain. 90 % of the immunized mice was protected against the lethal influenza virus

challenge. Recently, Firsov et al. (2015) successfully expressed M2e peptide in the form of M2e- β -glucuronidase fusion protein in *Agrobacterium*-transformed duckweed. The team expressed 30 amino acids long N-terminal fragment of M2 protein, containing 24 amino acid long fragment encoding M2e, optimized for expression in duckweed and fused upstream of the β -glucuronidase gene, under the control of CaMV 35S promoter. Stably transformed plants were obtained (nuclear transformation) with the highest yield of M2e- β -glucuronidase fusion protein being 1.89 and 1.96 % TSP (0.82 and 0.97 mg/g fresh weight, respectively, Firsov et al., 2015), accounting for app. 40 μ g/g of fresh weight of M2e alone. The protein accumulated in cytoplasm. The expression levels were similar to those previously obtained in experiments using virus-based transient expression systems, which is especially attractive as it could make duckweed a promising candidate for large scale production of influenza vaccine with regards to being able to produce equal amounts of protein than transient expression system at lower cost.

3 BACTERIAL VACCINES

3.1 *Escherichia coli* heat-labile enterotoxin subunit B

Enterotoxigenic *Escherichia coli* (ETEC) is the leading cause of diarrhea in developing world, causing severe mortality and morbidity rates especially in children up to 5 years of age in the developing countries. Enterotoxigenic strains of *E. coli* produce either the heat-stable toxin (ST), the heat-labile toxin (LT) or both. The structure and function of LT are highly similar to those of the cholera toxin. The B subunit of LT (LT-B) is non-toxic and works as a potent mucosal immunogen therefore it has been used as a target in developing candidate vaccines against ETEC-caused diarrhea as well as against cholera toxin, because the anti-toxin response caused by immunization with LT-B also extends to CT-B. Since the first LT-B subunit was expressed in potato and tobacco (Haq et al., 1995) many plant expression models have been developed and tested: tobacco leaf (Haq et al., 1995; Wang et al., 2001; Kang et al., 2006a; Chia et al., 2011), potato tubers (Mason et al., 1998; Tacket et al., 1998; Lauterslager et al., 2001),

maize seeds (Streatfield et al., 2001; Chikwamba et al., 2002), tobacco chloroplasts (Kang et al., 2004a), tobacco mosaic virus infected *N. benthamiana* plants (Wagner et al., 2004), transgenic corn (Tacket et al., 2004), *Siberian ginseng* somatic embryos (Kang et al., 2006b), carrot (Rosales-Mendoza et al., 2007; 2008), soybean seed (Moravec et al., 2007), lettuce leaf (Kim et al., 2007; Martinez-Gonzales et al., 2011), *Peperomia pellucida* tissue culture (Loc et al., 2010), watercress (Loc et al., 2011a) and transgenic tomato fruit (Loc et al., 2011b; Loc et al., 2014). In general, these models proved that synthetic non-toxic LT-B can be produced in plants without losing its native antigenicity or immunogenicity. Obtained yields ranged between 0.3 and 3 % TSP, with the highest yield of 3.3 % TSP in tobacco (Kang et al., 2006a) and 3.7 % TSP in maize seed (Chikwamba et al., 2002) and the lowest reported yields of 0.001 % TSP in the first transformed tobacco leaf and potato (Haq et al., 1995), 0.05 % TSP in lettuce leaf (Martinez-Gonzales et al., 2011) and 0.095 % TSP in tobacco leaf (Wang et al., 2001). As in the case of other

plant-made vaccines it has been proven that the addition of protein targeting sequences such as ER SEKDEL and the use of plant-preferred codons contributes to higher expression and accumulation of LT-B in plant tissue and most of the research is focused on searching for plant species that would express a sufficient amount of LT-B and could deliver it to the patient in raw unprocessed form. LT-B expressed in lettuce leaves (Martinez-Gonzales et al., 2011) elicited both serum and intestinal Ab responses in orally immunized mice, even in the case of freeze-dried lettuce tissue, with elicited antibodies showing neutralizing activity against cholera toxin challenge. Mice immunized with 8 µg of LT-B were fully protected against CT in the same way as mice immunized with the pure yeast-produced LT-B. Lettuce-derived LT-B assembled into pentameric forms, similar to the watercress-derived LT-B (Loc et al., 2011b). LT-B expression in lettuce was deemed stable as expression levels in T1 generation were similar to those in T2. Plant-derived LT-B has been found to be immunogenic and protective in orally immunized mice models for a majority of abovementioned plant expression models. In addition to that, LT-B expressed in potato tubers (Tacket et al., 1998) and transgenic corn (Tacket et al., 2004) was also immunogenic, protective and well tolerated in orally immunized humans. LT-B expressed in corn seeds (Karaman et al., 2006) elicited both mucosal and systemic immune responses in both young and aged mice and boosting by oral administration or injection of LB-T dramatically increased IgA and IgG levels in aged mice, which could be an indicator of immunological memory assembly. When expressed in transgenic tobacco chloroplasts, the LT-B accumulation was app. 2.5 % TSP, app. 250-fold higher than in plants generated by nuclear transformation (Kang et al., 2004). In addition to being expressed independently, LT-B is often expressed as an adjuvant for co-administered antigens, one of such experiments was carried out by Chia et al. (2011), expressing a fusion protein of PRRSV GP5 and LT-B in transgenic tobacco and its immunogenicity was evaluated in orally immunized pigs. It was found that the GP5-LT-B-treated pigs developed PRRSV-specific Ab- and cell-mediated immune response, but their ratios were not significantly higher than those observed in GP5-immunized pigs. Loc et al. (2014) recently expressed LT-B in the fruits of *Agrobacterium-*

transformed tomato. LT-B was expressed in the form of pentamers in the fruits in 2/5 transgenic tomato plants but thus obtained LT-B pentamers specifically bound to GM1 ganglioside, confirming their biological activity, which gives hope for tomato-produced LT-B as a suitable candidate for new-age subunit vaccine, however *in vivo* immunogenicity studies need to be performed.

3.2 Cholera toxin subunit B

Cholera is a highly epidemic diarrheal disease caused by the cholera toxin of enterotoxigenic *Vibrio cholerae* strain, affecting especially children in developing countries. The pentameric B subunit of the cholera toxin, CT-B, is structurally and functionally very similar to LT-B and has thus been the main target for developing plant-produced candidate vaccines against cholera as well as traveler's diarrhea. CT-B has been expressed in potato (Arakawa et al., 1997; He et al., 2007; Mikschofsky et al., 2009), tobacco (Daniell et al., 2001; Kang et al., 2004b, Kang et al., 2006c; Mishra et al., 2006; Mikschofsky et al., 2009; Tiwari et al., 2009; Rattanapisit et al., 2013), carrot (Kim et al., 2009), tomato leaves and fruit (Meena et al., 2002; Jiang et al., 2007; Loc et al., 2011b), lettuce (Huy et al., 2011), rice (Soh et al., 2015) and maize seeds (Karaman et al., 2012). As it is a potential mucosal and parenteral adjuvant and an effective carrier for chemically or genetically linked antigens, CT-B has often been expressed in the form of fusion proteins with antigens which do not induce sufficient immune response when orally applied on their own, much like LT-B. Examples of such use are CT-B- neutralizing epitope of the porcine epidemic diarrhea virus fusion protein (sCTB-sCOE) in lettuce (Huy et al., 2011), CT-B-rabies glycoprotein fusion protein expressed in tobacco seeds (Tiwari et al., 2009), CT-B- *Vibrio cholera* accessory colonization factor subunit A (ACFA) fusion in tomato (Sharma et al., 2008), fusion of simian-human immunodeficiency virus regulatory sequence and CT-B (Kim and Langridge, 2004b), simian immunodeficiency virus Gag p27 capsid protein-CT-B fusion (Kim et al., 2004a) and CT-B- anthrax lethal factor fusion in potato leaf (Kim et al., 2004b), CT-B- human insulin B chain in tobacco (Li et al., 2006) and CT-B- rotavirus enterotoxin NSP4 fusion protein in potato tubers (Yu and Langridge, 2001). Expression levels of CT-B in most plant systems were 0.01 - 1 % TSP, with the lowest yield of

0.04 % TSP in tomato leaf and fruit (Jani et al., 2002) and the highest yield of 4 % TSP in tobacco leaf (Daniell et al., 2001). In the case of CT-B-fusion proteins, the expression levels were lower, ranging 0.002-0.2 % TSP, the lowest obtained expression being 0.0003 % FW in potato tubers (Yu and Langridge, 2001) and the highest expression of 0.4 % TSP in tobacco (Roy et al., 2010). In general, expression of synthetic CT-B with plant-optimized codons, additional protein targeting sequences and driven by powerful plant promoters, was much higher than expression of other CT-B forms. When CT-B was expressed under the control of 35S CaMV promoter, using plant-optimized codons and additional ER SEKDEL retention signal its expression increased 10-fold, from previously obtained 0.081 % TSP (Jiang et al., 2007) to 0.9 % TSP (Loc et al., 2011b). Some of the plant-produced CT-B have been evaluated for their immunogenicity and protection in mice models, applied either orally or by gavage and it has been found that CT-B was immunogenic in all cases and has also induced protection in the form of both mucosal and systemic immune response (Jian et al., 2007; Huy et al., 2011; Karaman et al., 2012) in most cases, while in the case of CT-B-NSP4 rotavirus fusion protein it induced passive immunity (Yu and Langridge, 2001). However it has to be noted that in most cases when CT-B was expressed in plant host systems, either on its own or as a part of fusion proteins, its immunogenicity was not evaluated. In 2012, Karaman et al. expressed a synthetic CT-B gene, driven by γ -zein promoter in transgenic maize seeds and evaluated its immunogenicity in orally immunized mice. The highest expression levels in T1 seeds was 0.0014 of total aqueous soluble protein (TASP) while it increased to 0.0197 % TASP in T2 seeds, suggesting that the CT-B expression could be enhanced through selective breeding to advanced generations. Obtained CT-B was found to be immunogenic in mice and induced both mucosal and systemic immune response as shown by fecal IgA and serum IgG against both CT-B and LT-B, however the effect of serum anti-CT-B IgG on LT-B is not as big as its effect on CT-B and both enterotoxins show significant cross-immunoreactivity (Soh et al., 2015). It was suggested that the combined application of CT-B and LT-B could be an efficient way of protection against both cholera and diarrhea (due to similar

enterotoxin structure and mechanism of action). Soh et al. (2015) co-expressed recombinant LT-B and CT-B in rice grain for the first time. Both genes were driven by constitutive globulin promoter, an *Agrobacterium*-mediated transformation was performed and the obtained transgenic plants were grown to maturity to obtain 5 generations of transgenic seeds. Expression levels in the 5th generation of homozygous seeds as evaluated by ganglioside-dependent ELISA were 3.4 ng/ug of TSP for LT-B and 21.3 ng/ug of TSP for CT-B. Obtained CT-B and LT-B were present in their native pentameric form and capable of inducing both mucosal and systemic immune responses in orally and intraperitoneally immunized mice. When applied intraperitoneally, a dose of 100 μ g rice-produced LT-B and CT-B induced LT-B and CT-B-specific IgG production with similar expression levels. When applied orally, rice-produced LT-B induced both mucosal and systemic IgA production. These findings suggest that mucosal and systemic immune response against both CT-B and LT-B is enhanced by co-administration of both antigens and strengthens the immune response in either orally or intraperitoneally immunized mice. This could be of help with developing edible vaccines against cholera and traveller's diarrhea in economically important crop species like rice and maize. CT-B and LT-B synergism could be further enhanced by breeding to advanced generations and the immunogenic and adjuvant abilities of the co-expressed fusion toxins in rice should be further evaluated in animal studies (Soh et al., 2015). Rattanapisit et al. (2013) have developed a method of rapid transient expression of CT-B in *N. benthamiana*, using geminiviral replicon system, *Agrobacterium*-mediated transformation and a plant-optimized CT-B sequence. The highest obtained CT-B expression level was app. 4 μ g/g fresh weight \sim 0.14 % TSP as obtained on day 4, which was much higher than CT-B level transiently expressed in tobacco (Wang et al., 2001). Expressed CT-B showed biological activity for binding on the GM1-ganglioside in GM1-ELISA and could be thus a promising candidate for adjuvant for mucosal vaccines. The study suggests that geminiviral system could be efficiently used for high level expression of CT-B and further optimized, especially with regards to possible side effect of gene silencing.

4 OTHER RECENT VACCINES

4.1 Artherosclerosis

As an effort to develop immunotherapeutic treatments against artherosclerosis, different vaccination strategies have been proposed during the last 20 years. Most of these strategies were focused on targeting apolipoprotein B100 (ApoB100) and cholesterol ester transferase protein (CETP), trying to elicit immune response capable of modulating either artherosclerosis-associated inflammatory reactions or other up-regulated physiological mechanisms leading to this medical condition. To date, several clinical trials on plant-based vaccines against artherosclerosis have been reported: CTEP-tetanus toxin fusion (rabbits), CETi-1 with tetanus toxin as an adjuvant (humans), RHSP65-CETP (rabbits), p45 with AIOH as an adjuvant (mice), CT-B-p210 (mice) (Salazar Gonzales and Rosales-Mendoza, 2013), with some kind of artheroprotection reported in all cases, mostly shown as IgG or IgA. With the aim of initiating the development of a plant-based artherosclerosis vaccine, Salazar-Gonzales et al. (2014) constructed a synthetic gene encoding a fusion protein consisting of CT-B, CETP and ApoB100 and expressed it in tobacco. Epitopes of both ApoB100 and CETP were fused at the C-terminal end of CT-B, creating CTB:p210:CETP under the control of CaMV 35S promoter. The total of 6 transgenic tobacco plants was obtained, all of them containing transgene. Chimeric CTB:p210:CETP was expressed in tobacco in correctly assembled pentameric form, at the highest yield of 10 µg/g of fresh leaf tissue as obtained from the lines with single copy transgene insert. GM1-ELISA and Western blot analysis proved that the protein retained the target antigenic determinants. Subcutaneous administration of the chimeric protein elicited humoral responses against CETP and ApoB100 epitopes as well as human serum proteins in mice. These findings evidenced for the first time that atherosclerosis-related epitopes can be expressed in plants retaining immunogenicity, which opens a new path in the field of molecular farming for the development of vaccines against atherosclerosis.

4.2 Ebola

In their recent work, Bhoo et al. (2011) used a geminiviral replicon system for production of Ebola immune complex (EIC) in *Nicotiana benthamiana*. Ebola glycoprotein GP1 was fused at the C-terminal end of the heavy chain of humanized 6D8 IgG monoclonal antibody, specifically targeting the linear epitope of GP1. When co-expressed via geminiviral vectors, heavy chain and 6D8 light chain resulted in assembled immunoglobulin. It was purified and its conformation was evaluated by CC1q binding assay, dynamic light scattering and size exclusion chromatography. When subcutaneously immunized, mice elicited anti-Ebola virus antibodies at levels comparable to those obtained with a GP1 VLP, which is promising for using plant-produced EIC as human vaccines. Recently, a combination of 3 anti-ebola antibodies, called ZMapp, has been expressed in transgenic tobacco by Californian company Mapp Biopharmaceuticals (ISIS ..., 2014). ZMapp, previously tested only in monkeys, was administered to two Americans, infected in Africa, but in the case of the one that survived, it was not sure if that was due to ZMapp or was due to the blood transfusion received before ZMapp. So far no official report on production or function of ZMapp has been released. It is known, however, that the genes for humanized anti-ebola monoclonal antibodies are introduced to plant (tobacco) tissue via plant viral vectors by method of magnification and that the final yield of mAb adds up to 1 % of the cytoplasmic protein of tobacco leaf.

4.3 Anthrax

Anthrax has recently gained importance due to its potential application as a bio-warfare agent, besides frequent natural outbreaks around the world, especially in Africa, Central Asia and South America. The first research towards producing a plant-based subunit oral vaccine against anthrax was done by Aziz et al. (2002), who expressed protective antigen (PA) in tobacco, transformed by *Agrobacterium*-mediated transformation method. The PA transgene was found successfully integrated into tobacco nuclear genome and the protein was expressed with a predicted molecular weight of 83 kDa. Cytotoxicity assay confirmed

the retention of its biological activity. CT-B-anthrax lethal factor fusion protein was expressed in transgenic potato (Kim et al., 2004b), assembled into oligomeric structures, resembling the native pentamers, its accumulation level being 0.00039 - 0.0018 % TSP. This proved the feasibility of edible plants, such as potato, for production of anthrax lethal protein and its theoretical delivery. Recently, Gornatala et al. (2014) expressed protective antigene (PA) in Indian mustard (*Agrobacterium*-mediated transformation) and in tobacco (plastid transformation), under the control of CaMV 35S promoter. Expression level was 0.3 - 0.8 % TSP in

mustard and 2.5 - 4 % in tobacco. Macrophage lysis assay showed 23 - 81 % lysis for PA derived from mustard and 80 - 97 % lysis for PA from tobacco samples. Both oral and intraperitoneal application of either mustard- or tobacco-produced PA resulted in protective immune response with high serum PA-specific IgG or IgA titers in mice. Co-administration of CT-B as an adjuvant by gavage enhanced the response, even when antigen doses were as low as 5 or 10 µg. PA-specific mucosal immune response was observed in orally immunized mice.

5 CONCLUSION

During the last five years a lot of progress has been made in the field of plant-made vaccines. Novel transformation methods and expression vectors have been applied to plant species that had already proven themselves fit for expressing potential vaccine antigens, trying to enhance the expression of transgenes, protein accumulation and stability as well as facilitate the purification process. Obtained increases in accumulation and well-preserved biological function of produced antigens provide a

promising basis for future development of plant-derived vaccines. Before such vaccines can be produced on industrial scale and broadly accepted, there is still a lot to be done in order to eliminate all possible risks they could pose towards environment and living creatures and gain public acceptance of their usage, however, a lot more research and development of the field can be expected in the following years.

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