Sergio Rosales-Mendoza *Editor*

Genetically Engineered Plants as a Source of Vaccines Against Wide Spread Diseases

An Integrated View

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To all my friends and colleagues for their inspiration and encouragement.

Preface

Vaccines have saved more lives than any other biomedical invention. During the last two decades, novel conjunctions of scientific disciplines have revolutionized vaccine design and production. In particular, plant genetic engineering, bioinformatics, and molecular immunology have led to a novel manufacturing platform named plant-based vaccines, which has opened new paradigms for vaccine development.

During the last two decades, this concept has been elevated from merely performing conventional plant transformation approaches and orally administering raw plant material to sophisticated expression and processing technologies. At present, a substantial advancement on several aspects of this technology has been achieved, resulting in cases that are near to be introduced into the market.

This book aims to provide an insight into the principles, evolution, and state of the art of plant-based vaccines through contributions from leading experts within academia. An integrated view is provided by means of analyzing the incidence of the distinct fields of knowledge that converge in this multidisciplinary task, which include plant biology, recombinant DNA technology, biorreactor engineering, and immunology.

Section I presents the basis of plant-based vaccines. In Chapter 1, a general description of the methodologies involved in the design, production, and evaluation of plant-based vaccine candidates is provided as an introductory outlook of this technology. Chapter 2 covers in detail the immunology aspects involved in the induction of immunoprotective responses, with emphasis in the mucosal immunization routes.

Section II contains 4 chapters considering the principles of plant-based recombinant protein expression modalities as a key aspect in the development of plant-based vaccines. Among these, transient viral-based and plastid expression approaches have led to improved yields, allowing viable dosage for many prototype vaccines. Chapter 6 describes the principles of bioreactor-based plant biomass production as a critical part for implementing full contained production systems, which represents an advantageous approach in terms of biosafety.

The following part, Section III, shows the potential of plant-based production systems for developing novel vaccine candidates against relevant diseases, with emphasis in those considered in advanced development stages. In this comprehensive review, concrete vaccine candidates against important diseases are analyzed in

6 chapters as an outlook of the most advanced vaccines based in the use of plants as expression hosts.

The final part of this book, Section IV, is devoted to the discussion of perspectives that arise in this field comprising research goals related on advancing in the characterization of oral vaccines, addressing critical parameters to meet the regulatory standards, such as safety, potency, and reproducibility, as well as putative new target diseases to be addressed under this technology. Identified scientific goals are expected to be advanced in the short term, allowing for higher yields and stability, a more detailed characterization, and, as the ultimate consequence, improved applicability.

The present book is intended to serve as an accepted guide and tool for teaching and research activities, facilitating the study of this rapidly developing technology. I thank all my colleagues and students whose time and effort constituted a relevant support in this project. Special thanks go to my brothers for their unconditional love and support during the process of editing this book.

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Chapter 1
Principles of Plant-Based Vaccines

Dania O. Govea-Alonso, Guy A. Cardineau and Sergio Rosales-Mendoza

Introduction

Vaccination was introduced into medicine by Edward Jenner in 1796, who used poxvirus isolated from cows to immunize James Phipps against smallpox. This intervention resulted in the induction of protection against this pathogen, leading to the introduction of the term vaccine (Jenner 1798, 1801). A century later, Louis Pasteur developed a live attenuated vaccine against rabies and established the following basic steps for vaccine development: isolation, inactivation, and injection of the causative organism. These initial approaches served as guidelines for the development of vaccines throughout the twentieth century, allowing for the protection against many lethal infectious diseases (Fraser and Rappuoli 2005). Conventional approaches led to great achievements such as the eradication of smallpox and the virtual disappearance of many diseases, including diphtheria, tetanus, poliomyelitis, pertussis, decreasing mortality, and also increasing life quality and expectancy. For decades, inactivation and attenuation were the first choice for vaccine production; however, the difficulty of propagating some pathogens in vitro, and the fact that even attenuation may result in unwanted immune responses or risk of developing the disease, led to the consideration of alternative approaches. In addition, the purification of specific antigens often failed to provide a protective vaccine candidate, since conventional methods usually led to the identification of not only the most abundant but also the most variable and less suitable antigens (Moriel et al. 2008).

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At present, the existing vaccines in the market can be categorized as (1) whole-celled killed formulations, which contain the causative agent inactivated by chemical/physical treatment; (2) whole-celled live attenuated formulations, containing the live causative agent, but unable to cause disease; (3) subunit vaccines, which are of highly defined composition comprising purified antigen(s) derived from the causative agent; and (4) conjugate vaccines, consisting of a polysaccharide component of the causative agent that is poorly immunogenic on its own and is thus chemically linked to a protein.

Since the invention of vaccination as a preventive approach for infectious diseases, the development of vaccines has dramatically advanced, and new trends in the field are currently directed to improve the vaccination benefits in terms of the number of targeted diseases, cost, safety, and global coverage.

A priority in the field involves applying recombinant DNA technology for the production of recombinant subunit vaccines as they are considered safer, since no pathogen is present, and can be scaled up more easily. These efforts have resulted in the exploration of several expression systems, including *Escherichia coli*, yeast, mammalian cells, and insect cells. However, in spite of expanding the vaccination coverage, lowering the production cost remains a challenge in the global vaccination arena. This is particularly critical for developing countries where the demand is highest but the access to preventatives and therapeutics is limited due to political, economic, and logistical issues (Drake and Thangaraj 2010; Penney et al. 2011).

In this context, the research community developed a new platform based on plant cells as biofactories for the production of biopharmaceuticals (Goldstein and Thomas 2004). Within this trend, plant-based vaccines were conceptualized as antigenic formulations derived from transgenic plant biomass expressing specific antigens, intended to serve as a vaccine. In 1990, the concept of plant-based vaccines was described and demonstrated for the first time in the publication of a patent application by Roy Curtiss, III and Guy A. Cardineau, who achieved the production of transgenic tobacco plants capable of expressing a colonization antigen of *Streptococcus mutans*. This plant-derived antigen was proposed as a means for eliciting secretory immune responses when orally administered in humans or animals, which could be capable of inhibiting colonization and/or invasion through the mucosal surface. This research resulted in the first patent related to the plant-based vaccine technology (Curtiss and Cardineau 1997, US 5,654,184).

In the early 1990s, three main groups were working to prove the concept of plant-based vaccines, and, in 1992, Charles Arntzen's group published the first peer-reviewed report consisting of the expression of hepatitis B surface antigen. After these pioneering studies, several groups adopted this focus and started the exploration of several antigens from distinct pathogens in order to assess the viability of this technology in a number of plant species, mainly tobacco, potato, tomato, lettuce, spinach, and corn. In this chapter, the principles of plant-based vaccines are presented and a general description of the development steps involved is provided.

Rationale of Plant-Based Vaccines

A plant-based vaccine formulation is intended to serve as a source of a recombinant antigen produced in a low-cost host, whose biomass or purified fractions are intended to serve as elicitors of protective immunity throughout the administration by distinct routes (Salyaev et al. 2010). This represents a promising strategy for the production of mucosally delivered vaccines, especially oral vaccines, which require minimal processing of the raw plant biomass and training for administration.

The mucosa is the major entry site for many pathogens, which invade the host through respiratory, gastrointestinal, or genital surfaces, eliciting a secretory immunoglobulin A (IgA) response to provide a first line of defense against those pathogens. Membrane surfaces are associated with a group of organized lymphoid tissue structures known as mucosa-associated lymphoid tissue (MALT). These can be subdivided into distinct terms according to anatomical localization, which include the gut-associated lymphoid tissue (GALT), the nasopharynx-associated lymphoid tissue (NALT), and the bronchi-associated lymphoid tissue (BALT). In the GALT, the main mucosal inductive sites include the Peyer's patches (PPs), a large cluster of lymphoid follicles. The follicle-associated epithelium (FAE) covering PPs contains the specialized antigen-sampling epithelial cells, the microfold (M) cells (Staats et al. 1994). These cells possess folded luminal surface and do not secrete digestive enzymes or mucus and has a thin (20 nm) glycocalyx surface that prevents the access of >1 μm particles (Takahashi et al. 2009). The functions of M cells comprise transport of intact macromolecules and microorganisms across the epithelial barriers to subepithelial dendritic cells (DCs) that may present those antigens in adjacent mucosal T cell areas. Importantly, M cells also present a pocket in the basolateral membrane, which is tightly associated with DCs and T and B lymphocytes. Thus, these pockets also serve as sites for the initiation of mucosal immune responses (Takahashi et al. 2009). Following antigen presentation, B cells migrate to distant effector sites, including the lamina propria (LP) of the gut and respiratory tract. As a consequence, dimeric IgA is produced and secreted, having the potential to prevent the initial interaction of the pathogen with host receptors or neutralize pathogen toxins, leading to protective immunity. Since PPs are also populated by serum IgG-producing cells, local IgG synthesis can be also elicited by mucosal vaccination (Mowat and Viney 1997).

One important feature of mucosal vaccines relies on the ability of stimulating both mucosal and systemic immune responses, providing two relevant arms to achieve immunoprotection. In addition, this form of delivery offers additional advantages as it does not require sterile devices such as syringe and needles for administration, making this practice more acceptable and decreasing the cost of global immunization programs. However, there are some drawbacks associated with this immunization route. For example, it is difficult to measure the effective dose for a mucosally delivered vaccine as it is exposed to the complex environment of the gastrointestinal tract. Further, oral vaccines may require coadministration with specific adjuvants to reach sufficient immunogenic activity (Mestecky et al. 2008). These and other immunological aspects will be reviewed in detail in Chap. 2.

Initially, the concept of plant-based vaccines envisioned the use of fresh or minimally processed plant tissues as a direct source of orally administered formulations (Curtiss and Cardineau 1997, US 5,654,184). However, in terms of dosage and stability, the view has evolved into one requiring some processing of the plant biomass to allow at least the production of a freeze-dried powder, which can be dosed properly and stored at room temperature for long periods of time (Alvarez et al. 2006). Such processed plant material may be compressed into tablets or used to fill capsules and is perhaps the most likely vehicle in which oral plant-based vaccines may reach the market.

Advantages of Plant-Based Vaccines

Important features of effective vaccines include safety, protective immunity, stability, ease of administration, low cost, and minimized side effects. Subunit vaccines have been developed and studied for decades and typically comprise bacterial polysaccharides or proteins, purified from pathogenic organisms. These pure subunits of pathogenic origin are safer than whole-celled vaccines since they lack replicative capacity; thus, the risk of reversion of attenuated strains or survival of putatively killed pathogens is avoided (Buetow and Korban 2000).

Currently, most subunit vaccines are produced in recombinant systems in which the antigen(s) responsible for the induction of protective immunity is genetically engineered for expression in a non-pathogenic host organism; these vaccines require purification and, as with other common vaccine preparations, the soluble product requires cold chain logistics in order to maintain activity during storage and transportation, thereby increasing the production costs (Pelosi et al. 2012).

Since subunit vaccines consist of small fractions of the pathogen, immunogenic properties are substantially modified with respect to those derived from whole cells. In general, immunogenicity is greatly decreased, which is reflected by the absence of high reactogenicity, constituting a desirable effect for some formulations. However, low immunogenicity can lead to weak immune responses, generating the need for coadministering adjuvants to attain immunoprotection (Liljeqvist and Ståhl 1999).

In addition to those advantages associated with conventional subunit vaccines, the use of plants for vaccine production represents the following convenient features:

- It constitutes the most economical and feasible source of recombinant products, resulting in a US$ 40 billion industry of new therapeutics and industrial enzymes (Howard 2005). This reduced manufacturing cost is due to the replacement of fermenters and bioreactors with contained plant growth rooms or green houses with appropriate biological containment (Daniell et al. 2005). It is estimated that

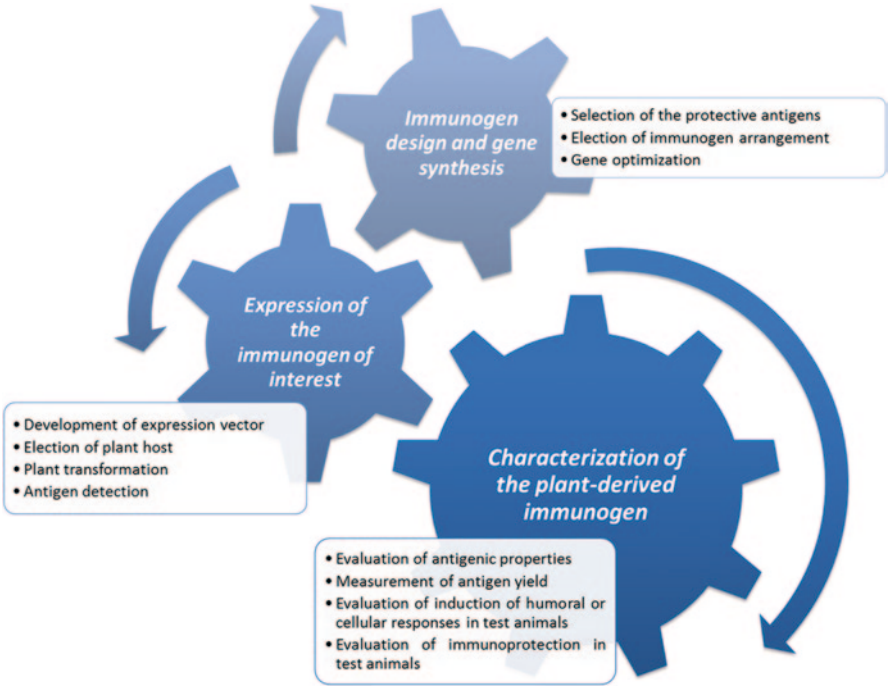

Fig. 1.1 Steps in the development of plant-based vaccines

costs for producing a recombinant protein in transgenic plants is 10–50-fold lower than producing it by means of *E. coli* fermentation (Giddings et al. 2000).

- Plants can properly produce complex foreign proteins, as these hosts possess expression, folding, assembly, and glycosylation machinery, which is associated with the preservation of immunogenic activity of vaccines (Wycoff 2005).
- Unlike mammalian systems, plant systems are not hosts for human or animal pathogens such as viruses or prions, and hence they do not transmit such pathogens.
- Many plant species can serve as safe oral-delivery vehicles; in particular, specific tissues such as grains, fruits, or leaves can allow for the formulation of vaccines without extensive purification and processing. These vehicles can be easily produced by a freeze-dried process leading to formulations with high stability under a cold chain-free distribution (Korban 2002).

The following section describes a general view of the steps involved in the development of plant-based vaccines (Fig. 1.1).

Steps Involved in the Development of Plant-Based Vaccines

Immunogen Design and Gene Synthesis

The first step in the development of a plant-based vaccine candidate consists of the selection of the protective antigens involved in the target pathogen/disease, which is aided by bioinformatics, genomics, and proteomics that offer the possibility of performing a rational design of antigenic proteins. At the end of the twentieth century, the design of most vaccines was ruled by traditional technologies. Remarkable progress has been attained by the introduction of new technologies such as recombinant DNA and chemical conjugation of proteins to polysaccharides, as well as advances in the use of novel adjuvants. Moreover, a powerful tool emerged when access to genomes of microorganisms was initiated by Craig Venter, who published the genome of the first free-living organism in 1995 (Fleischmann et al. 1995). This technological revolution allowed moving beyond conventional approaches by means of using software and databases to accomplish rational design vaccines without the need for growing the specific microorganisms. This new approach is denominated "reverse vaccinology" (Sette and Rappuoli 2010).

For a given pathogen, immunoprotective epitopes can be identified by reliable assays such as those used in the isoltaion of MHC–epitope complexes, and phage display technology (Rueckert and Guzmán 2012; Dormitzer et al. 2008). Assessment of the immunoprotective potential of the elected epitopes or antigens require the following resources: (1) a well-annotated genome sequence of the pathogen under investigation, (2) an efficient platform for heterologous protein expression starting from the elected gene, and (3) a robust model, which truly mimics human infection and/or immunological mechanisms that, in humans, correlate with protection (Grandi and Nagy 2012).

Further analyses allow for the confirmation of the immunoprotective effects of the proposed candidates. For example, synthesizing overlapping peptides can allow for measuring their immunogenic activity by means of in vitro assays where peripheral blood mononuclear cells of exposed or vaccinated donors are stimulated with these peptides.

In parallel with epitope- and antigen-mapping studies, it is important to conduct additional studies to further validate the role of the humoral and cellular responses in immunity and protection. Knowing which epitopes are presented by infected cells, as opposed to cross-presented, may be critical to determine vaccine design. This has been addressed in the case of the vaccinia virus (VACV) system by examining the kinetics of antigen presentation in conditions favoring cross-presentation versus recognition of infected target cells (Gasteiger et al. 2007; Moutaftsi et al. 2006). Additional studies have analyzed the protective capacity of different VACV epitopes and found that the best correlates of protective capacity were high immunogenicity and capacity of being presented by infected cells (Moutaftsi et al. 2009).

Fortunately, a large set of data on immunogenic epitopes exist in the literature for a myriad of pathogens; thus, this fact greatly facilitate the formulation of new vaccine candidates in a relatively straightforward approach.

Of special interest is the design based on highly immunogenic carriers that allow for the elicitation of effective immune responses to unrelated antigens. Typical carriers comprise the B subunit of the enterotoxins produced by enterotoxigenic *E. coli* or *Vibrio cholerae*. These have a singular capacity of serving as potent mucosal immunogens. On the other hand, virus-like particles (VLPs) are self-assembling structures that can also incorporate specific unrelated epitopes through genetic fusion, thus serving as a particulate delivery system (see Chap. 3). One important feature of these particles is given by their high immunogenicity that allows for the induction of immune responses even at very low doses at the nanogram scale (Soria-Guerra et al. 2011).

Once the immunogen design has been completed, the next step involves the design and synthesis of a transgene encoding for the elected antigenic protein. For these purposes, a number of companies offer the gene synthesis service. Current approaches for gene synthesis are most often based on a combination of organic chemistry and molecular biology techniques, allowing for the production of entire genes without the need for precursor template DNA. This methodology has become an important tool in many fields of recombinant DNA technology, including vaccine development, gene therapy, and synthetic biology.

Important parameters to consider in the design of synthetic genes include the following: inclusion of flanking restriction sites to facilitate the molecular cloning procedures required to construct expression vectors, matching the codon bias with that of the expression host, and removal of undesired introns or unstable RNA motifs, thus optimizing gene expression in the specific host (Gustafsson et al. 2004; Hoover and Lubkowski 2002).

Expression of the Immunogen of Interest

Establishing an approach to achieve the plant-based expression of antigens comprises the development of a specific expression vector, choosing a plant host, and performing plant transformation.

Among the key elements in the expression vector, promoters mediate the transcriptional activity of the expression cassette (Walden and Schell 1990). Proteins or subunit vaccines can be produced in plants by expression cassettes driven by constitutive promoters or, alternatively, by inducible or specific promoters if the protein should be selectively expressed in a particular tissue or organ in order to maximize accumulation or avoid deleterious effects on the plant host. For example, seed- specific promoters can enable recombinant proteins to be accumulated at convenient levels within the plant seed. This concept has been proven in the case of corn and rice, claiming a number of advantages such as high yields, facilitated long-term storage at ambient temperature and convenient edible material for vaccine formulations (Hefferon 2012). Seed-based approaches are analyzed in detail in Chap. 5.

On the other hand, transcription machineary can also be engineered to favor expression. It has been reported, for example, that T7 RNA polymerase expressed from the nuclear plant genome enhances the expression of a transgene in the context of plastid-based expression (Magee and Kavanagh 2002).

Untranslated regions (UTRs) also play an important role in the transgene expression efficiency. The 5′ UTR is an important element that may influence the translational efficiency. At the same time, the 3′ UTR region plays an important role in gene expression as it contains signals for transcript polyadenylation that directly influence mRNA stability (Sharma and Sharma 2009).

Additional regions in the expression vector comprise expression cassette-flanking regions, which mediate homologous recombination events. These sequences are critical when site-specific integration of the expression cassette is pursued, which is the typical objective for the chloroplast transformation approaches (Rosales-Mendoza et al. 2008). A deeper insight into these transplastomic approaches is provided in Chap. 4.

At the technical level, a synthetic gene, which is typically provided in a cloning vector, should be released by appropriate restriction enzymes and subsequently subcloned into the elected expression vector. After ligation reaction, the construct should be confirmed by restriction profile analysis and sequencing to ensure the open reading frame (ORF) integrity. It is important to mention that an advantageous trend is directed at homologous recombination, which consists of using site-specific recombination events in order to perform facilitated and accurate cloning procedures (Karimi et al. 2002; Earley et al. 2006).

To date, many plant expression vectors are commercially available. For nuclear expression using *Agrobacterium tumefaciens* as the transformation delivery system, binary vectors that are replicative in both *E. coli* and *A. tumefaciens* are typically used. On the other hand, viral- and plastid-based expressions require particular designs, which are presented in detail in Chaps. 3 and 4, respectively.

Selection of a particular plant species as expression host is an elemental choice with critical implications on the vaccine to be produced. Earlier, tobacco and potato were the systems of choice for production of many plant-based recombinant proteins, essentially due to the easiness with which these can be genetically modified (Horsh et al. 1985). This approach was very useful to start with, proving the concept of a number of candidate vaccines. However, to date, a large number of plant species are being used for this purpose, including maize, carrot, tomato, soybean, lettuce, potato, and alfalfa. These models offer particular advantages related to better yields and absence of toxic compounds, making possible oral immunization using raw plant materials.

The choice of the plant species should be based on the specific objective that is pursued. Some of the factors influencing this choice include expression strategy, the life cycle, biomass yield, containment, and scale-up cost (Sharma and Sharma 2009). *Nicotiana* species are the most popular choice for transient expression approaches due to the high biomass yield and easy growth (Ma et al. 2003). However, edible crops are ideal when the development of an oral vaccine is pursued. Some of the edible crops that have been frequently used include lettuce, carrot, tomato, corn, and rice, among others (Fischer et al. 2004).

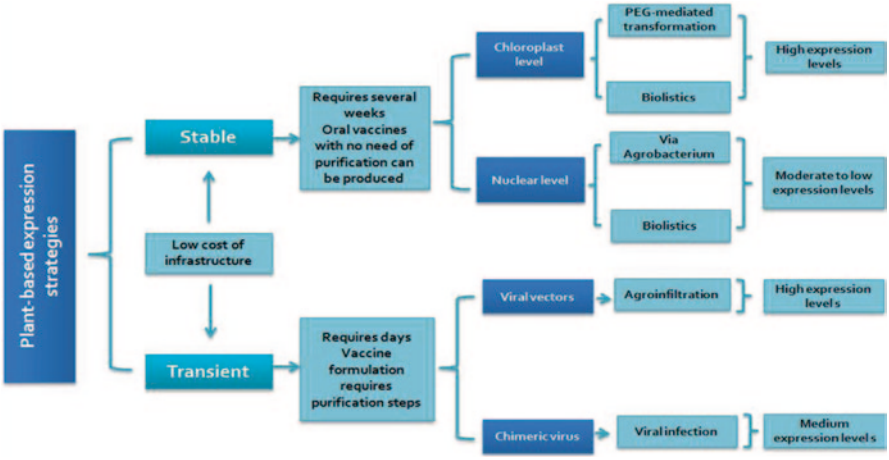

Fig. 1.2 Summary of the strategies for the plant-based expression

Once a host has been elected, distinct strategies can be accomplished for the plant-based expression of the target antigen, which essentially can comprise stable or transient expression approaches applied to whole plants, plant tissues, or cell suspension cultures (Fig. 1.2).

The stable transformation can be achieved at nuclear or chloroplast level. The first step in plant transformation is the introduction of the desired foreign expression cassette into the target plant cell. This can be performed by different methods that are chosen according to the host plant or the type of tissue to be transformed. Some of the widely used methods include electroporation, biolistics, or the use of biological vectors such as *Agrobacterium* or viruses. Currently, *Agrobacterium*-mediated transformation is the method of choice for the nuclear stable transformation of most of the plant species due to its simplicity and capability of introducing large segments of DNA with minimal rearrangement, higher efficiencies with low number of insertions, and low cost. However, once the foreign DNA is in the nucleus, the integration occurs randomly; therefore, positional effects may influence the expression of the foreign protein or cause undesirable phenotypic characteristics. Another phenomenon that can take place under these approaches is silencing. Since some plant species are recalcitrant to *Agrobacterium*-mediated transformation, alternative methods can be applied, such as biolistics or protoplast PEG-mediated transformation (Rao et al. 2009).

In the case of the production of transplastomic plants, the transgene can be introduced by biolistics or protoplast PEG-mediated transformation, and the expression vector typically targets the insertion in a site-specific manner by means of double homologous recombination mediated by appropriate flanking sequences (Gómez et al. 2009; Tiwari et al. 2009). Chapter 4 of this book provides a thorough analysis of the transplastomic approaches.

When stable transformation approaches are pursued, plant tissues are subjected to a regeneration process following the gene transferring procedure. The objective of this step is to favor the proliferation of the transformed cells through a selective condition given by the presence of a selective agent according to the employed selectable marker. Therefore, only successfully transformed cells are able to yield whole plants. In vitro conditions direct the regeneration processes since morphogenetic response of the tissue is determined by plant growth regulators and culture conditions (light, temperature, etc.). These conditions are often optimized for the elected plant. Two pathways for plant regeneration are widely followed: somatic embryogenesis and organogenesis.

In somatic embryogenesis, development of whole plants from somatic cells occurs in a manner analogous to development of plants from zygotic embryos. These embryos can be produced directly or indirectly. In the direct somatic embryogenesis process, the embryo is formed directly from a cell or group of cells without the production of an intervening callus, while in the indirect somatic embryogenesis callus is first produced from the explants (Pathi et al. 2013).

Organogenesis refers to the production of organs, either directly from an explant or from a callus culture. Organogenesis relies on the inherent plasticity of plant tissues and is regulated by altering the components of the medium. Typically, the auxin-to-cytokinin ratio of the medium determines what developmental pathway the regenerating tissue will follow. It is well established that shoot formation is induced by increasing the cytokinin-to-auxin ratio in the culture medium (Slater et al. 2008).

On the other hand, transient expression strategies imply the expression of a foreign DNA which cannot be inherited, but is transcribed within the host cell in a temporary manner. This approach constitutes a convenient tool that overcomes the difficulties associated with stable transformation and offers the advantage of the rapidity with which protein yield is achieved, since typically whole plants are used, thus avoiding regeneration steps. The use of the plant virus approach relies on the fact that viruses can infect the plant, producing a systemic infection, generating multiple copies of the genome. *Tobacco mosaic virus* (TMV)-based expression vectors are the most widely used vectors for the production of foreign proteins in plants. Leaves can be harvested after few weeks post infection, followed by antigen purification. These kinds of approaches have achieved prominent productivity in the field of producing vaccines in plant cells (Gleba et al. 2004, 2005). This topic is analyzed in detail in Chap. 3.

A set of molecular and biochemical parameters should be evaluated in the transformed plants. In particular, for the transgenic approaches, the first screening is conducted by polymerase chain reaction (PCR) and Southern blot techniques in order to assess the presence of the transgene. Phenotype is also described for the transgenic lines as phenotypic alterations are a possibility when expressing a heterologous protein.

Characterization of the Plant-Derived Immunogen

Once the transgenic state of the elected lines as well as the expression of the expected recombinant protein are determined, it is necessary to quantify the amount

of protein produced per gram of fresh or dry weight. This objective is typically accomplished by enzyme-linked immunosorbent assay (ELISA) and western blot assays. A critical step in determining the potential of a specific vaccine candidate comprises the immunogenicity and immunoprotective capacity. In the preclinical level, test animals are subjected to a defined immunization scheme in order to determine whether or not it is capable of inducing a specific immune response when administered under the elected route and dosage. Antibody levels and proliferation of specific immune cells are often evaluated by in vitro methodologies such as ELISA and splenocyte proliferation assays.

In addition, crucial evidence of the vaccine potential consists of assessing the protection against a specific pathogen challenge. For this purpose, a pertinent animal model susceptible to the pathogen of interest should be identified and used to assess the potential for preventing the development of the disease. This parameter can be evaluated by scoring of deaths in vaccinated and unvaccinated test animal groups or by measuring disease-associated parameters. Once verified that the candidate vaccine induces humoral and/or cellular immune responses and has immunoprotective potential and acceptable safety in test animals, clinical trials are considered viable.

Subunit vaccine candidates produced by plants or plant viruses have been extensively assessed in preclinical trials. Immune responses have been recorded with several of these vaccine candidates administered by various routes, including intraperitoneal, subcutaneous, intramuscular, intranasal, or oral routes. Among these evaluations, the delivery of minimally processed plant tissues is of key importance for the development of oral vaccines. Focusing on an approach that avoids antigen purification is considered the priority in the field (Yusibov et al. 2011). Early studies centered on feeding mice highly immunogenic molecules such as the B subunits of the heat-labile toxin and cholera toxin expressed in plant tissues; however, many of the candidates remain to be characterized in this sense (Rosales-Mendoza et al. 2008).

Another important parameter involves analyzing the elicitation of cell-mediated immunity, since only a small number of candidates have been tested for immunogenicity in humans. To date, clinical trials utilizing transgenic plants for vaccines have comprised either the leaves or fruits from the plants (Lugade et al. 2010). The prototype plant-based vaccines for human pathogens that have garnered the most clinical data are the enterotoxigenic *E. coli* (ETEC), *Norwalk virus, Influenza virus, Rabies virus, and Hepatitits B virus* (Tacket et al. 1998, 2000; Thanavala et al. 2005; Yusibov et al. 2011). Chapter 13 of this book presents a view in depth on this matter.

In conclusion, the development of plant-based vaccines has been established and matured over the last two decades. Tools allowing these developments have yielded distinct strategies that can be applied to pursue the assessment of specific candidates. This chapter has provided a general view of the steps involved in plant-based vaccine development, while subsequent chapters aim to present a wider view of each of the aspects of this emerging and relevant research field.

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Chapter 2
Mucosal Immunology and Oral Vaccination

Ana Lilia García-Hernández, Néstor Rubio-Infante and Leticia Moreno-Fierros

Introduction

The mucosal surfaces of the gastrointestinal and respiratory tracts represent the principal portals of entry for most infectious agents. Hence, the development of vaccination strategies capable of inducing protective immune responses at the mucosal sites is a priority. Since the mucosal surfaces are exposed to a wide variety of antigens, the mucosal immune system has to discriminate between harmful and harmless inoffensive or beneficial antigens. For this reason, the mucosal immune surfaces are highly regulated by a complex interplay of regulatory mechanisms capable of eliciting strong immune responses against pathogens and protecting the body as well as preventing the induction of strong immune responses against dietary proteins, commensal bacteria, or environmental inoffensive antigens, which can lead to chronic diseases (Mowat 2003; Pabst and Mowat 2012).

Mucosal surfaces are protected from external attacks by physicochemical defense mechanisms comprising innate and adaptive mucosal immune systems. Epithelial barriers on the mucosal surfaces at different sites in the body differ dramatically in their cellular organization, and antigen-sampling strategies at diverse mucosal sites are adapted accordingly. The intestinal mucosa is covered by only a single cell layer (type 1 epithelium), whereas multilayered squamous epithelia line the oral cavity, pharynx, esophagus, and urethra (type 2 epithelium); and the airway and vaginal linings vary from pseudo-stratified to simple epithelium (Box 2.1; Pavot et al. 2012).

A major goal in vaccine design comprises the induction of protective lasting immune responses against potential pathogens on the mucosal surfaces. These responses are most effectively induced by the administration of vaccines onto mucosal surfaces through oral, nasal, rectal, or vaginal routes, when compared with those induced by parenteral routes (Neutra and Kozlowski 2006). In addition, mucosal vaccines offer

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Box 2.1 Mucosal Immunity Is Mediated by Different Lines of Defense

(1) IgA, antimicrobial peptides (such as defensins, angiogenins, defensin-like peptides, and catelicidins released by enterocytes, Paneth cells, as well as by intraepithelial lymphocytes), and mucus glycoproteins
These components are the first line of defense forming a mucosal layer and dismiss the penetration of most bacteria. IgA neutralizes pathogens while antimicrobial peptides can reach sufficient levels to mediate bacterial lysis in crypts (Mowat 2003).
(2) Epithelial barrier
The second barrier of defense comprising the monolayer of the epithelial cells (ECs) and the upregulated permeability provided by tight junctions through these cells, which are formed by a single epithelial stem cell; absorptive enterocytes, microbicidal factor-producing Paneth cells, mucus-producing goblet cells, and hormone-producing enteroendocrine cells protect against invasion of luminal microbes into the sterile tissues (Brandtzaeg et al. 1999).
(3) Lamina propria
It is considered the final barrier before systemic immunity and contains distinct lymphoid structures that can detect and restrain microbes through the action of dendritic cells, macrophages, lymphoid cells, stromal cells, and plasmatic cells (Coombes and Powrie 2008).

needle-free delivery, thereby improving accessibility, safety, and cost-effectiveness. Mucosal vaccines are also advantageous when compared with systemic vaccines from a production and regulatory perspective. For example, vaccines for oral use do not require extensive purification from bacterial by-products since the gut is already heavily populated by bacteria, whereas the same vaccine formulation injected parenterally would have unacceptable endotoxin levels (Lycke 2012). Nevertheless, the vast majority of vaccines in use today are administered by intramuscular or subcutaneous injections, where a proper control on dosage can be accomplished. By contrast, the dose of a mucosal vaccine that enters the body is not accurately determined. Moreover, several challenges to achieve successful mucosal vaccination still prevail, comprising poor induction of mucosal immunity, limited understanding of protective mechanisms and cross talk between mucosal compartments, and the availability of safe and effective mucosal adjuvants as well as delivery systems. Our understanding of mucosal immunity and development of mucosal vaccines has lagged behind, in part because the induction and measurement of mucosal immune responses are more complicated than those elicited by parenteral routes. As a result, only a few mucosal vaccines have been approved for human use worldwide. Among these, oral vaccines against poliovirus, *Salmonella typhi, Vibrio cholerae,* and rotavirus, and a nasal vaccine against influenza virus can be mentioned (Pavot et al. 2012; Woodrow et al. 2012). However, research and testing of mucosal vaccines are currently accelerating, stimulated by new information on the mucosal immune system and by the threat of the mucosally transmitted virus, such as the Human

immunodeficiency virus (HIV). Fortunately, current research is providing new insights into the function of mucosal tissues and the interplay of innate and adaptive immune responses that result in immune protection at mucosal surfaces (Neutra and Kozlowski 2006).

To better understand the limitations and challenges for developing successful oral vaccines, some general anatomical and functional characteristics of the mucosal immune system will be described in this chapter, particularly of the one associated with the intestinal mucosa. Current strategies for successful mucosal vaccination will be further analyzed, highlighting the advantages of oral vaccines.

Organization of the Mucosal Immune System

The mucosal immune system can be divided into inductive and effector sites. The first ones are constituted by organized mucosa-associated lymphoid tissue (MALT) as well as mucosa-draining lymph nodes. The latter are represented by the lamina propria (LP), the stroma of exocrine glands, and surface epithelia.

MALT comprises multiple compartments including the gut-associated lymphoid tissue (GALT), which is the largest human mucosa and immunologic organ in the body. The gastrointestinal mucosa is associated to specialized components of the innate and adaptive immunity (specific antigen recognition, effector and memory functions) that protect the host against pathogens, control responses to food components, and mediate tolerance against harmful antigens (Holmgren and Czerkinsky 2005).

In the GALT, the organized tissues responsible for the induction phase of the immune response comprise the Peyer's patches (PP) and mesenteric lymph nodes (MLNs), as well as smaller, isolated lymphoid follicles (ILFs), which have the appearance of microscopic PP and are distributed throughout the walls of the small and the large intestines. The diffuse lymphoid tissue of the effector sites at the intestinal mucosa consists of lymphocytes scattered throughout the epithelium and LP of the mucosa (Fig. 2.1).

Characteristics of the Organized Inductive Lymphoid Tissues

Organized lymphoid tissues such as the PP consist of collections of large B cell follicles and intervening T cell areas. The lymphoid areas are separated from the intestinal lumen by a single layer of columnar epithelial cells, known as the follicle-associated epithelium (FAE), and a more diffuse area immediately below the epithelium, known as the subepithelial dome (SED; Fig. 2.1). The FAE differs from the epithelium that covers the villus mucosa as it has lower levels of digestive enzymes and a less pronounced brush border, and it is also infiltrated by large numbers of B

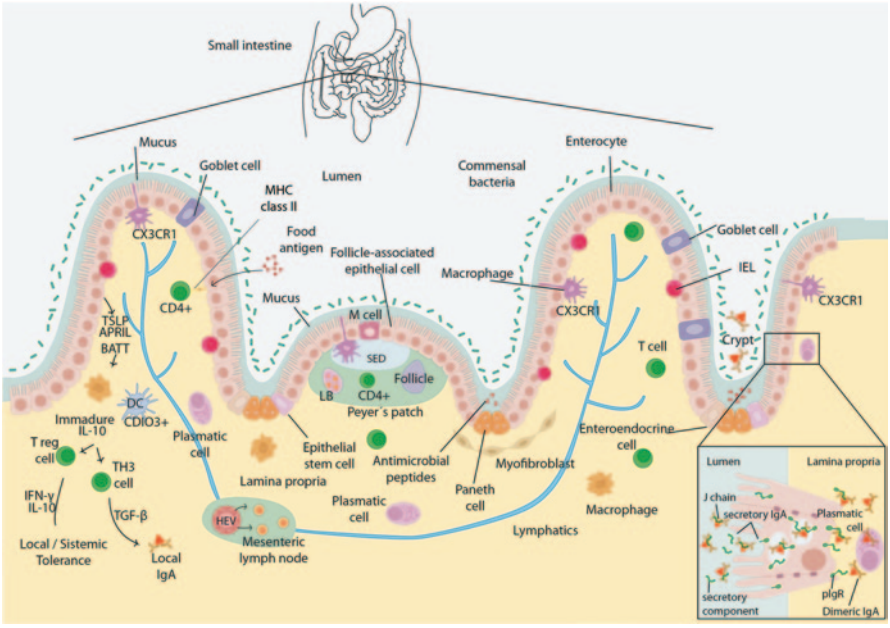

Fig. 2.1 Anatomy and homeostasis of the intestinal immune system. The gut-associated lymphoid tissue (GALT) can be divided into inductive and effector sites, which consist of organized and diffuse lymphoid tissues, respectively. The organized tissues are the Peyer's patches (PP) and mesenteric lymph nodes (MLNs), as well as smaller, isolated lymphoid follicles. The effector tissues consist of lymphocytes scattered throughout the epithelium and lamina propria (LP) of the mucosa. A single layer of intestinal epithelial cells (IECs) provides a physical barrier that separates the commensal bacterial in the intestinal lumen from the underlying LP. The IECs lining the lumen are bathed in nutrients, commensal bacteria, IgA, and goblet cell-produced mucus. These IECs differentiate into villous or colonic enterocytes, which absorb nutrients (small intestine) and water (colon). Progenitor IECs differentiate into both enteroendocrine cells, which secrete enteric hormones, and Paneth cells at the base of the small intestinal crypts. Paneth cell granules contain high concentrations of α-defensins. Certain subsets of T cells (intraepithelial lymphocytes, IEL) and macrophages cells CX3CR1+ localize between the IECs. In the small intestine, about 80% of IEL are CD8+ lymphocytes and about 70% of CD4+ lymphocytes is present in the LP. The specialized epithelium termed follicle-associated epithelium contains microfold (M) cells that overlie the subepithelial dome (SED) of the organized lymphoid tissue PP consist of a rich zone of B lymphocytes in an area termed follicles, and around them is a thymus-dependent area (TDA), which is rich in CD4+ T lymphocytes. The LP, contains B cells (especially sIgA-producing plasmatic cells), T cells CD4+, stromal cells, and antigen-presenting cells (APCs) such as macrophages and dendritic cells (DCs) CD103+. Oral tolerance is essential to maintain homeostasis. Food proteins and products of commensal bacteria are taken up by IECs which express MHC II, but do not express the co-stimulatory molecules; thus, they contribute to oral tolerance induction. IECs also produce chemokines like APRIL and B-cell-activating factor (BAFF), which promote B cell recruitment in the LP and class switching in response to TLR signaling, and thymic stromal lymphopoietin (TSLP), the transforming growth factor-β (TGF-β), retinoic acid (RA), and possibly other factors that promote the induction of regulatory T (Tregs) cells. Specific subsets of intestinal DCs CD103+ express RA-synthesizing enzymes, and in the presence of TGF-β, induce the differentiation of naïve T_R cells, Foxp3+. RA also programs DCs to imprint gut-homing properties. These committed T_R cells home back to the intestinal LP through high endothelial venules (HEVs), where they undergo secondary expansion under the influence of interleukin-10 (IL-10) produced by CX3CR1+ macrophages. These T cells differentiate into Treg cells, and also produce IL-10 and interferon-γ (IFN-γ) and/or T helper (T_H) 3 cells, which produce TGF-β-favoring oral tolerance

cells, T cells, macrophages, and dendritic cells (DCs). The most notable feature of the FAE is the presence of microfold (M) cells, which are specialized enterocytes that lack surface microvilli and the normal thick layer of mucus. Antigens are taken up by absorptive epithelial cells or specialized epithelial M cells in mucosal inductive sites, or alternatively, can be directly captured by "professional" antigen-presenting cells (APCs), which include DCs, B lymphocytes, and macrophages. Antigen-charged DCs further process and present antigens to T cells located at the interfollicular areas within the PP. Primed lymphocytes exit through the draining lymphatics to the MLNs, where they reside for an undefined period of further differentiation before they migrate into the bloodstream through the thoracic duct and finally accumulate in the mucosa (Holmgren and Czerkinsky 2005; Mowat 2003).

Priming of T and B cells in these inductive tissues and selective homing to mucosal sites lead to either efficient local immune responses or tolerance. However, how the intestinal captured antigens can also induce systemic priming or tolerance involves complex mechanisms. The MLNs are considered alternative sites where T cell priming might occur and explain the induction of local and systemic immunity or tolerance by the oral route. The antigens might reach the MLNs via the draining lymph (Fig. 2.2) or as a result of APCs located in the LP that have taken up antigens either directly from the lumen or from APCs that have acquired unprocessed antigens from M cells, and then migrated to MLNs. T cells that are primed in the MLNs are further differentiated, and then migrate to the mucosa to mediate local immune responses. In addition, since the MLNs can act as a crossover point between the peripheral and systemic immune systems, this pathway might also explain the induction of systemic immunity or tolerance in response to intestinal antigens (Mowat 2003).

Mucosal Effector Tissues

The diffuse lymphoid tissues are mainly associated with effector responses that are initiated from the organized lymphoid tissues. These diffuse lymphoid tissues are mainly composed of lymphocytes residing as intraepithelial lymphocytes (IELs) in the mucosal epithelium in addition to numerous lymphocytes present in the LP, which is the connective tissue directly underlying the mucosal epithelium.

Intraepithelial Lymphocytes

The IELs that reside within the epithelium of the intestine form one of the main branches of the immune system by their direct contact with the enterocytes and by their immediate proximity to antigens in the gut lumen. As IELs are located at this critical interface between the core of the body and the outside environment, they must balance protective immunity with an ability to safeguard the integrity of the epithelial barrier, as failure of this function would compromise homeostasis (Cheroutre et al. 2011).

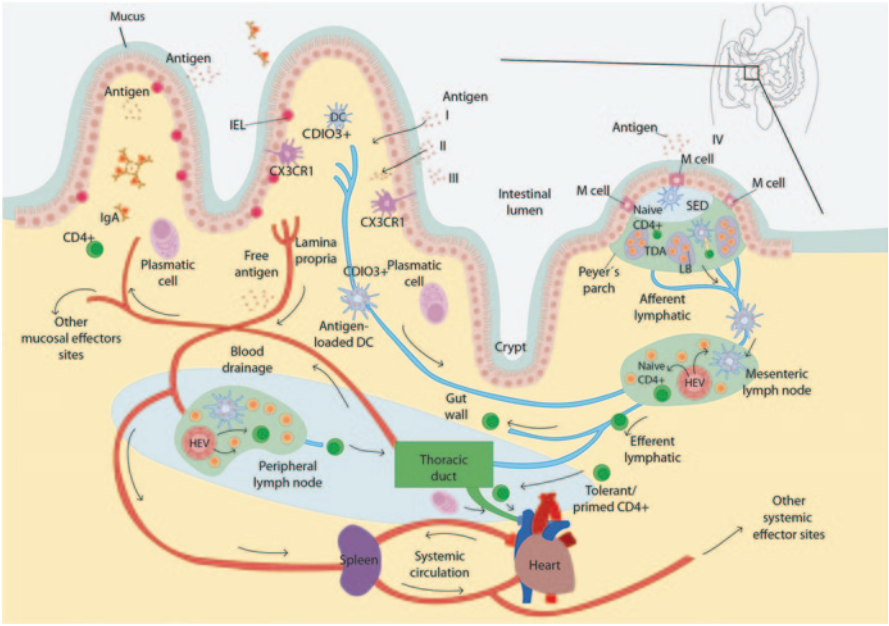

Fig. 2.2 Antigen uptake in gut-associated lymphoid tissue (GALT). The antigen might enter GALT through different parts of the intestine. Epithelial cells can acquire soluble antigens that have diffused through epithelial tight junctions (*I*) or have been transferred across epithelial cells by transcellular routes (*II*). CX3CR1+ macrophages can also capture luminal antigens by extending processes through the epithelial layer, and they may pass this to neighboring CD103+ dendritic cells (DCs) (*III*). Also, the antigen might enter through the microfold (M) cells in the follicle-associated epithelium (FAE) (*IV*) and after transfer to local CD103+ DCs; the antigen might also gain direct access to the bloodstream from the gut and interact with T cells in peripheral lymphoid tissues. The antigen taken up into Peyer's patches (PP) or lamina propria may enter the bloodstream via the portal vein, first reaching the liver before it becomes distributed into the circulation. Free antigen taken up into afferent intestinal lymph will pass through the mesenteric lymph nodes and eventually enter the bloodstream via the thoracic duct. Once the antigen is sampled by M cells, it is delivered across the epithelial barrier directly to subepithelial DCs that subsequently process and present antigen locally to T cells located at the interfollicular areas within the PP. Alternatively, antigen or antigen-loaded DCs from the PP might gain access to the draining lymph, with subsequent T, B cell recognition in the mesenteric lymph nodes (MLNs). In all cases, the antigen-responsive CD4+ T cells or plasmatic cells acquire expression of the α4β7 integrin and the chemokine receptor CCR9, leave the MLN in the efferent lymph, and, after entering the blood stream through the thoracic duct, exit into the mucosa through the vessels in the LP. T cells and plasmatic cells, which have recognized antigen first in the MLN, may also disseminate from the bloodstream throughout the peripheral immune system. Plasmatic cells produce local sIgA and systemic IgG. Since T cells and plasmatic cells migrate through the circulation, integrin and chemokine signals direct their emigration into tissues. In this manner, imprinted T cells and plasmatic cells have a specific key that allows access to restricted tissues

IELs essentially comprise antigen-experienced T cells belonging to both T cell receptor-γδ (TCRγδ)$^+$ and TCRαβ$^+$ lineages, but are extremely heterogeneous, and the various IEL subsets are distributed differently in the epithelia of the small and large intestines probably influenced by the distinct digestive functions and the physiological conditions between both intestines. In the small intestine, IELs are almost exclusively T cells and include a significant proportion of TCRγδ$^+$ cells (60%). IELs constitutively express CD103 (also known as the αE integrin), which interacts with E-cadherin on intestinal epithelial cells, and most of them, especially in the small intestine, express CD8αα homodimers, which is a hallmark of their activated phenotype. The majority of IELs express activation markers, such as CD44 and CD69; contain abundant cytoplasmic granules responsible for cytotoxic activity; and can express effector cytokines, such as interferon-γ (IFNγ), interleukin-2 (IL-2), IL-4, or IL-17. Furthermore, IELs characteristically express both activating and inhibitory types of innate natural killer (NK) cell receptors, which typify them as stress-sensing (activated) yet highly regulated (resting) immune cells (Cheroutre et al. 2011). IELs play an important role in controlling the entrance of commensal bacteria after epithelial damage via the release of antimicrobial peptides and promoting the repair of injured gut epithelia. IELs express a limited diversity of antigen receptors, keep in a heightened state of activation, and thus avoid the need for a priming step before full activation.

Lamina Propria Lymphocytes

Lymphocytes in the LP include mainly the CD4+ T cells and also an important population of plasma cells, which are B lymphocytes that are mainly IgA in type I mucosal tissues like the one present in intestines. An important characteristic of the mucosal adaptive immune response is the local production and secretion of dimeric secretory immunoglobulin A (sIgA), which, unlike other antibody isotypes, are resistant to degradation in the protease-rich external environments of mucosal surfaces. sIgA is secreted as a dimer across the mucosal epithelium by an active transport mechanism using the polymeric Ig receptor (pIgR). sIgA has multiple roles in mucosal defense as it can bind and neutralize pathogens or toxins in the gut despite the presence of active digestive enzymes. It promotes the entrapment of antigens or microorganisms in the mucus preventing direct contact of pathogens with the mucosal surface, a mechanism that is known as "immune exclusion." Protection of mucosal surfaces by sIgA can also be mediated by intracellular neutralization of pathogens that have invaded the epithelial cells when the sIgA is transported by the pIgR. In addition, antigens can be excreted through the secretion of sIgA joined to the antigens, which is released into the mucosal lumen (Strugnell and Wijburg 2010). Moreover, sIgA-mediated blockade is also a key element in the intestinal homeostasis as it reduces inflammatory activity of the microbiota (Mantis et al. 2011).

Although the adaptive humoral immune defense at mucosal surfaces is mainly mediated by sIgA, locally produced IgM and IgG in the respiratory tract and in the

genitourinary mucosa and serum-derived IgG can also contribute significantly to the mucosal immune defense (Neutra and Kozlowski 2006; Iwasaki 2010).

The lymphocytes that enter the mucosa redistribute into distinct compartments. The functions of mucosal T cells are still largely undefined, but cells with a "memory" or "effector memory" phenotype predominate in both the epithelium and the LP, indicating that these have been exposed to an antigen. In the LP of the intestine, CD4+ T cells are of particular importance in regulating local immune responses. LP CD4+ T cells might be regulatory T (Tregs) cells and therefore responsible for maintaining local tolerance to environmental antigens. These produce large amounts of cytokines, particularly IFN-γ, but also IL-4 and IL-10.

LP CD8+ T cells can also have potent cytotoxic T lymphocyte (CTL) activity. Some of these antigen-experienced LP T cells might be true effector cells, and might help local B cells to produce IgA "effector memory" cells, as indicated by the findings supporting that antigen-specific memory CD4+ and CD8+ T cells accumulate preferentially in non-lymphoid tissues, particularly at the intestinal mucosa (Shale et al. 2013; Mowat 2003).

Intestinal CD4+ T cells are essential mediators of immune homeostasis and inflammation. Multiple subsets of CD4+ T cells have been described in the intestine, which represents an important site for the generation and regulation of cells involved in immune responses both within and outside of the gastrointestinal tract. Among intestinal lymphocytes, CD4+ T cells represent a major population implicated in mediating diverse host-protective and homeostatic responses (Shale et al. 2013).

T cell populations can be broadly functionally divided into effector and regulatory populations. The lack of inflammation in the majority of individuals, despite the enormous microbial and antigenic load within the intestine, clearly demonstrates the dominance of regulatory mechanisms in the steady state, condition in which IL-17 cells are the dominant Th17A single positive CD4+ T cells, and preferentially locate the LP of the small intestine and, to a lesser extent, the colon and intestine of adult mice. Interestingly, expansion of Th17 cell populations in the small intestine may occur in the setting of extraintestinal infections or autoimmune diseases without detectable mucosal inflammation. In the steady state, the presence of dominant, suppressive, and regulatory mechanisms restrains innate and adaptative responses. Functionally specialized subsets of CD4+ T cells play an important role in the regulation of intestinal immune responses. The concept of an important functional role for CD4+ Treg cells in maintaining intestinal homeostasis was established originally in mice, where the ability of CD4+ CD25+ Treg cells to prevent disease in the T cell transfer model of colitis was described. A number of subsets of T cells possessing regulatory or suppressive activity have now been characterized, but those expressing the transcription factor Foxp3 and IL-10-producing cells appear to be of particular functional importance in intestinal homeostasis and in the control of inflammation. In comparison with systemic immune compartments, the intestine is enriched with the presence of Treg cells. Although IL-10+ Foxp3+ Treg cells are also found in abundance in the small intestinal LP, a sizable fraction of IL-10+ CD4+ T cells in this location do not express Foxp3, exhibiting a Tr1 phenotype. TGF-β plays a critical role in the development and function of Treg cells, including

Foxp3 + and Tr1 cells. Cells co-expressing RORγτ and Foxp3 are found in intestinal tissues. Notably, both Treg cells and Th17 cells require TGF-β for their development, and the presence or absence of further factors, including the STAT3-activating cytokines, IL-6 and IL-23, may determine the balance of these populations in the steady state or inflamed intestine. Interestingly, intestinal CD4 + T cell subsets are also regulated by environmental factors. The microbiota directs the accumulation of both Treg cells and Th17 cells in the intestinal LP (Shale et al. 2013).

Intestinal APCs

Together with the epithelial barrier, APCs and IELs are located at the first line of defense. After sensing pathogens, these cellular types release cytokines, antimicrobial peptides, and chemokines as defense or activate and recruit immune cells that furthermore can phagocytose and kill pathogens.

Antigen sampling strategies are adapted to the diverse epithelial barriers that cover mucosal surfaces throughout the body, but all involve collaboration with APCs. Myeloid APCs of the intestine are a heterogeneous population consisting of DCs and macrophages (Pabst and Mowat 2012; Geissman et al. 2010; Scott et al. 2011; Manicassamy and Pulendran 2009). These populations are strategically positioned with the LP and in organized lymphoid structures, and exhibit a number of adaptations associated with their dual role in tolerance and immunity in the intestine. Myeloid APCs might congregate immediately under epithelia, migrate into the epithelial layer, and even extend dendrites into the lumen to capture antigens. DCs can act as a bridge with the adaptive immune system through their ability to acquire antigen in the intestine and migrate to the MLN where they prime the activation of cognate naive T cells. In addition to presenting antigens, intestine-derived DCs are specialized in their ability to prime T cell responses that are focused on the intestine through the upregulation of gut-homing molecules on the responding T cell surface (Box 2.2).
At sites of organized mucosal lymphoid tissues, specialized M cells in the lymphoid FAE sample and deliver antigens across the epithelial barrier directly to subepithelial

Box 2.2 Importance of Mucosal Homing in the Choice of Mucosal Vaccination Route

After the initial exposure to antigen, lymphocytes leave PP or other mucosal inductive sites and migrate into mucosal tissues, including the intestines, lungs, nasal passages, and urogenital tract. These lymphocytes home to the LP or mucosal epithelium where they exert effector activities such as antibody synthesis or killing virally infected cells. The preferential migration of mucosal stimulated lymphocytes to other mucosal sites throughout the body gave rise to the idea of a “common mucosal immune system.” However, it is now apparent that the mucosal immune system is highly compartmentalized

and thus lymphoid responses preferentially migrate into tissues where the response was induced. Therefore, the compartmentalization within the mucosal immune system places constraints on the choice of vaccination route for inducing effective immune responses at the desired sites (Holmgren and Czerkinsky 2005). Therefore, in order to induce and regulate protective immune responses at the appropriate mucosal sites, depending on the invading route of a particular pathogen, it is required to understand the biological basis of the mucosa compartmentalization (Brandtzaeg et al. 1999).

The capacity for selective migration of effector and memory T and B cells back to the original challenge site—the concept of tissue-specific homing—or to the distinct mucosal sites, depends on the differential expression of adhesion molecules on the lymphocyte cell surface as well as on the vascular endothelium. Whereas naive T cells express adhesion molecules and chemokine receptors that restrict their migration mainly (but not entirely) to organized lymphoid tissue, activated memory T cells downregulate these lymphoid-tissue-homing receptors and upregulate tissue-specific adhesion molecules and chemokine receptors that target their migration to non-lymphoid tissues (Kunkel and Butcher 2002).

This imprinting of tissue-homing properties is best described for the gut and skin. Priming of T and B cells in PP and mesenteric lymph nodes preferentially induces the expression of α4β7 integrin and CC-chemokine receptor 9 (CCR9), whereas T cells that are primed in peripheral lymph nodes upregulate cutaneous leukocyte antigens, CCR4 and CCR10. Endothelial cells of postcapillary venules in the intestinal mucosa constitutively express ligands for α4β7-integrin and CCR9, namely mucosal addressin cell-adhesion molecule 1 (MADCAM1) and CC-chemokine ligand 25 (CCL25), also known as thymus-expressed chemokine (TECK), which is expressed selectively by small bowel epithelial cells, allowing lymphoid cells that are induced in intestinal lymphoid tissue to enter this mucosal effector site. Importantly, recent investigations suggest that antigen-presenting DCs process and "interpret" locally produced metabolites to program tissue-specific lymphocyte homing. In the case of GALT, resident DCs metabolize vitamin A to retinoic acid, which stimulates α4β7-integrin and CCR9 expression by T cells; and in the skin, local DCs use metabolites of vitamin D3 to program T cells in recurrent laryngeal nerves (RLNs) (Kunkel and Butcher 2002).

The identification of α4β7-integrin and CCR9 as mucosal homing receptors interacting with MADCAM1 and CCL25, respectively, was considered the molecular explanation for the fact that mucosal vaccination is required for protection against mucosal infections, whereas parenteral vaccines are generally ineffective to induce mucosal immunity. It must be taken into account that recruitment of lymphoid cells into target tissues requires specific chemokine recognition and adhesion-receptor engagement. The high degree of compartmentalization among the distinct mucosal sites relies on the use of distinct

set of mucosal homing receptors. Indeed, there are distinct tissue-trafficking patterns for both B and T cells that depend on their site of induction. For example, plasma-cell precursors that are primed in respiratory tract lymphoid tissues home to the tracheal and bronchial mucosa, express only low levels of the gut-homing molecules, α4β7-integrin and CCR9, but express high levels of α4β1-integrin and CCR10. Importantly, the counterparts of α4β1-integrin and CCR10, vascular cell adhesion molecule 1 (VCAM1) and CC-chemokine ligand 28 (CCL28), respectively, are constitutively expressed by airway mucosal endothelial cells. Lung T cells also express distinct phenotypes and lack intestinal-homing molecules. Moreover, nasal and vaginal cells also express a phenotype that is distinct from gut-homing T cells (Iwasaki 2010; Holt et al. 2008).

DCs that subsequently present antigen locally in adjacent mucosal T cell areas. Gut APCs can be found scattered in the LP of the gastrointestinal tract (small and large intestine) and in the radial muscle layer. The intestinal DCs play the most important role as professional APCs, since they express up to 100 times more major histocompatibility complex (MHC) molecules and are more effective in priming naïve T cells in the organized lymphoid tissue (PP and ILFs), MLN, and LP. DCs are considered the primary inductors to T-cell-dependent IgA responses (Manicassamy and Pulendran 2009). In mice, APCs can be grouped based on the expression of CD103 (α-E integrin) and CX3CR1 (the receptor of fractalkine). CX3CR1+ cells derive from a monocytic precursor that may be recruited in response to the microbiota and have been described to drive the development of Th17 cells in vitro, presumably via a flagellin or ATP-dependent pathways. In particular, CX3CR1+ CD11b+ CD11c+ DCs and CD103+ DCs have been well characterized, while in homeostatic conditions CD103+ DCs derive from circulating DC precursors (pre-DCs) and have tolerogenic potential. These cells can also imprint T cells with gut-homing properties in both mice and humans (Scott et al. 2011). Regarding CX3CR1+ APCs, it is not yet clear what their function is, as they are incapable of migrating out of the gut and low-effective APCs (Pabst and Mowat 2012). However, a recent report has shown that these cells acquire migratory properties in the absence of the microbiota.

Routes of Antigen Uptake and Induction of Mucosal Immune Responses

The immunological consequences of oral administration of antigen ultimately depend on where and how antigen is taken up and presented to T cells. Most soluble, non-adherent antigens are taken up at low levels, and generally induce immune tolerance (Pabst and Mowat 2012).

The possible routes of antigen uptake are outlined in Fig. 2.2. The conventional pathways by which it is assumed that this might occur comprise the uptake of particulate antigen into PP or isolated lymphoid tissues through M cells, and also by

the alternative routes of antigen uptake which might be of relevance. These are the following: the transfer of intestinal antigen and/or APCs from the PP or mucosal LP through the draining lymph to the MLNs, followed by local presentation to naïve T cells; blood-borne dissemination of antigen to peripheral lymphoid tissues; transfer of antigen to the liver through the portal vein; and local presentation of antigen to T cells by enterocytes or professional APCs in the LP (Pabst and Mowat 2012).

Villus enterocytes participate in a route for antigen uptake and also have been proposed as intestinal APCs directed to CD4+ T cells. This function was proposed since enterocytes are MHC class-II positive in most species, but normally do not express the co-stimulatory molecules that are required for full T cell activation; thus, they were considered as good candidates for tolerogenic APCs in vivo. However, it is improbable that presentation of antigen by enterocytes to adjacent CD4+ T cells might help to explain local tolerance, since naive CD4+ T cells are located in the organized lymphoid tissues and are rarely located in the LP. In addition, LP T cells do not migrate out of the gut, and, therefore, it seems unlikely that this pathway could contribute to systemic tolerance (Mowat 2003).

Role of Epithelial Cells in Mucosal Defense

In the gastrointestinal tract, a single layer of epithelial cells joined by tight junctions faces a complex luminal environment rich in microorganisms. Epithelia and their associated glands (such as the salivary glands) produce non-specific or innate defenses, including mucins and antimicrobial proteins. Nevertheless, foreign antigens and microorganisms frequently breach the epithelial barrier, and mucosal tissues are sites of intense immunological activity. In the intestinal mucosa, dispersed lymphoid cells and APCs are particularly abundant. Epithelial cells are active participants in mucosal defense since they function as sensors that detect dangerous microbial components through pattern recognition receptors such as Toll-like receptors (TLRs), and they respond by sending cytokine and chemokine signals to the underlying mucosal cells, such as DCs and macrophages, to trigger innate, non-specific defenses and promote adaptive immune responses. In the intestine, where bacteria are abundant, epithelial cells, together with IELs and the underlying phagocytic cells, can modulate and dampen these signals to prevent undesirable responses to non-threatening nutrients and the normal intestinal flora that could lead to mucosal inflammation (Artis 2008; Rescigno 2011).

Regulation of the Intestinal Immune System and Oral Vaccination

The mucosal surfaces are continually exposed to a wide variety of foreign antigens. As many of them do not represent any risk to the body, such as food proteins and commensal bacteria, maintaining the homeostasis and preventing damage or mucosal disorders, such as allergy and mucosal inflammatory diseases, are accomplished by sophisticated regulatory mechanisms that had evolved at these sites. The gastrointestinal tract is the largest reservoir of immune cells in the body; thus, the intestinal immune system is also considered the most complex part of the immune system. The mucosal immune system is able to distinguish between pathogenic and commensal bacteria or other inoffensive antigens and mount the appropriate immune responses, either effective protective immunity or regulatory responses. For example, protecting the gastrointestinal tract from invading pathogens requires strong protective immunity. By contrast, active immunity against non-pathogenic materials would be wasteful, and hypersensitivity responses against dietary antigens or commensal bacteria can lead to chronic inflammatory disorders such as coeliac disease and inflammatory bowel disease, respectively. Therefore, the default responses to most soluble non-toxic antigens are either mucosal immune tolerance or non-inflammatory responses (Pabst and Mowat 2012).

Particularly, the usual response to harmless gut antigens consists of the induction of local and systemic immunological tolerance, known as oral tolerance (Mowat 2003). In addition to its physiological importance, this phenomenon can be exploited for the treatment of autoimmune and inflammatory diseases, but it is also an obstacle when the development of recombinant oral vaccines is pursued. For these reasons, understanding the processes that determine the immunological consequences of oral administration of antigens is of key importance.

Basis of Tolerance Induction at the Mucosal Tissues

It has been proposed that specific features of mucosal tissues favor the induction of tolerance in terms of production of IgA antibodies and, to a lesser extent, T helper 2 (Th2) cell responses. However, several features of mucosal tissues might contribute to these effects, including a unique ontogeny and anatomical patterning, specialized cells and organs that are involved in the uptake of antigen, distinctive subsets of APCs, and several unusual populations of B and T cells. In addition, the migration of lymphocytes to the intestine is controlled by a series of unique adhesion molecules and chemokine receptors (Pabst and Mowat 2012).

Challenges in Oral Vaccine Design and Current Strategies to Achieve Mucosal Immune Responses

Mucosal vaccines that are orally administered face the same gauntlet of host defenses as do microbial pathogens: They are diluted in mucosal secretions, captured in mucus gels, attacked by proteases and nucleases, and excluded by epithelial barriers, and thus relatively large doses of vaccine are required, and it is difficult to determine with accuracy the dose that crossed the mucosa (Neutra and Kozlowski 2006).

Several strategies have been developed to advance the development of mucosal vaccines, including the use of diverse antigen-delivery systems and mucosal adjuvants. The main characteristics of these strategies, including advantages and limitations, are summarized in Table 2.1 (see Box 2.3).

Box 2.3 Routes of Mucosal Vaccination

Nasal route

Intranasal administration is an attractive immunization route due to the following features: Nasal mucosa is a practical site that lacks acidity, the secreted enzymes are limited, and small mucosal surface area requires a low dose of antigen. Furthermore, the nose is highly vascularized, easily accessible, and can be used for global immunization of large populations. It is well established that vaccines administrated by nasal route can induce both mucosal and systemic immune responses, preferentially if the vaccine is based on attenuated live cells or an antigen is accompanied by an adjuvant. This has been confirmed in nasal immunization of humans against diphtheria, tetanus, influenza, and *Streptococcus mutans*. Furthermore, potent responses in the respiratory and genital tracts can be induced by intranasal immunization as a result of the induction sites in nasopharynx-associated lymphoid tissue (NALT) that contains all of the immunocompetent cells required for the induction of antigen-specific immune responses. Nasal vaccination has proven to be an effective regimen for the stimulation of the respiratory immune system and can elicit both humorla and cellular responses.

Different nasal vaccine systems in humans and animals have been described. In fact, an intranasal live influenza virus vaccine has been approved by the Food and Drug Administration (FDA). This vaccine is safe, well tolerated, and up to 93 % effective against culture-confirmed influenza (Rappuoli et al. 2011; Woodrow et al. 2012; Pavot et al. 2012; Cheroutre et al. 2011; Yuki and Kiyono 2009).

Vaginal route

Vaginal mucosa is characterized by a type II epithelium that does not have histologically demonstrable MALT, but these mucosal surfaces in the female genital tracts are protected by distinct epithelial cell layers, mucus, and by distinct innate and adaptive effector mechanisms. Specific immune cells in genital mucosae comprise intraepithelial T cells, macrophages, Langerhans cells (LCs), and submucosal DCs present in type II epithelia of the vaginal canals, which provide immune protection (Iwasaki 2010).

After infection, innate cells, including monocytes, neutrophils, NK cells, and plasmacytoid DCs (pDCs), are mobilized to the vaginal tissue. In the steady state, LCs in the epithelium and DCs in the submucosa are highly phagocytic and express high amounts of PRRs. After pathogen recognition through PRRs, DCs and LCs undergo a maturation program and migrate to the draining lymph nodes to prime naive T and B cells. At a later time point, antigen-specific T and B cells enter the tissue to provide pathogen-specific immune defense. Due to the absence of inductive sites (MALT) in vaginal mucosa, priming of lymphocytes occurs exclusively in the draining lymph nodes including the common iliac, interiliac, external iliac, and inguinal femoral lymph nodes. Delivery of vaccines by genital routes is not very practical in human trials due to many disadvantages, comprising the cumbersome administration of a mucosal vaccine through the genital tract as well as the immunological features of the female reproductive tract due to hormonal fluctuations during the menstrual cycle (Kozlowski et al. 2002)

Oral route

The elicitation of immune responses in the intestinal mucosa by an orally administered antigen comprises its transportation by different pathways:

(1) Through M cells that are present in the follicle-associated epithelium of the PP or located in ILFs. Basolateral membrane of M cells is heavily invaginated while the apical one has little glycocalyx, presumably aiding antigen uptake, which is then captured by DCs, permitting their maturation and migration to the intrafollicular areas. M cells possess a high transcytotic capacity and are able to transport a broad range of materials. This pathway preferentially occurs for particulate antigens (Holmgren and Czerkinsky 2005; Neutra and Kozlowski 2006).

(2) Directly from the lumen by CX3CR1+ macrophages, (3) across epithelial cells, or (4) through epithelial tight junctions. The uptaken antigen can be transferred to CD103+ DC within the PP or in the lamina propria directly by these cells. The APCs process the antigen and migrate within the PP to the T cell areas and/or B cell follicles (inductive sites). T follicular helper (TFH) cells subsequently co-localize with B cells in the B cell follicle in close proximity to a follicular dendritic cell (FDC) network, and this allows the formation of a germinal center where the antigen-specific B cells undergo class-switching to IgA and somatic hypermutation to generate higher-affinity antibodies. Free antigen or antigen-loaded DCs from the PP or LP might gain access to draining lymph, with subsequent T cell recognition in the MLNs resulting in the induction of mucosal and systemic effector immune responses of T cells and B cell-producing IgA or IgG antibodies. The resulting IgA+ long-lived plasma cells and memory B cells generated within the germinal center leave the PP through the efferent lymph and migrate to the MLN and subsequently to the blood through the thoracic duct. Plasma cells home to bone marrow and to effector sites in the lamina propria of the small and large intestine. MLNs can act as a crossover point between the mucosal and systemic immunity and explain the induction of systemic immunity induced by intestinal antigens (Mowat 2003).

Table 2.1 Advantages and limitations of the distinct immunization routes

	Advantages	Limitations
Oral	Delivery: ingestion. Recipient-friendly approach	Requires mucosal adjuvant
	The delivery risk is minimal; no syringes or needles required	Required high antigen dose. Digestion in the gastrointestinal tract. Efficient uptake of particulate antigens
	Elicited responses: humoral and cell immune response. Mucosal IgA in large and small intestines, vagina, and salivary gland induce modest systemic antibody, and CTL responses	Can induce tolerance
	Extensive use for attenuated vaccines. Against rotavirus, *Vibrio cholera, Salmonella typhi,* and poliovirus	Limited clinical trials of subunit vaccines
	Is the safest route of vaccine delivery	
Nasal	The delivery risk is minimal; no syringes or needles required	Requires delivery devices. Requires full cooperation of the vaccinee
	Efficient antigen transfer across nasal epithelium	Requires mucosal adjuvant
	Elicited responses: systemic antibody and mucosal IgA in large intestine, vagina, and nasal cavity; CTL responses	Requires medium antigen dose
		Can induce tolerance
		Limited number of clinical trials. Against influenza
		Evidence of antigen transfer to neuronal tissue via olfactory bulb in mice. Clinical studies indicate that Bell's palsy is caused by influenza nasal vaccine that contains the native form of heat-labile *Escherichia coli*
Parenteral	Requires low antigen dose	Delivery: injection Requires medically trained personnel
	Potent systemic antibody and T cell	Possible transmission of infection by contaminated needles and syringes
	Extensive clinical use in many viral, bacterial, and parasitic diseases	Alum most widely used as adjuvant, but a variety of systems are effective
	Elicited responses: no major problems with subunit vaccines	Null response in mucosa
		Mild-to-serious side effects with killed or attenuated vaccines

Oral delivery of non-living vaccines has proved to be extremely challenging, owing to poor stability of proteins, peptides, and DNA in the acidic and enzyme-rich environments of the gastrointestinal tract. Several strategies, including the use of biodegradable polymeric particles and liposomes, had been adopted to protect antigens in the gastrointestinal tract. In addition, strong adjuvants, for example, enterotoxins

such as cholera toxin (CT) and the heat-labile enterotoxin from enterotoxigenic *Escherichia coli* (LT), have been successfully used for the oral immunization of test animals. However, toxicity of these enterotoxins limits their applications in humans. To alleviate the toxicity issues, mutants and subunits of LT and CT have been used as adjuvants in many studies of oral immunization in animals with some promising perspectives (Box 2.4; Lycke 2012; Martin et al. 2000). Table 2.2 presents an overview of adjuvants and delivery vehicles developed for mucosal immunization.

Box 2.4 Adjuvants and Antigen-Delivery Systems

Intestinal immune system is tightly regulated and polarizes the immune response mainly to tolerogenic responses; thus, the development of new strategies for the enhancement of optimal immune response is urgently needed. Strong adjuvants such as bacterial enterotoxins (CT or LT) have been successfully used for oral immunization in mice. Recently, a rice-based vaccine that expressed CTB subunit has proved to serve as an effective long-term cold chain-free oral vaccine that induces CTB-specific sIgA-mediated long-standing protection against *V. cholerae* or LT-ETEC-induced diarrhea. As CTB lacks enzymatic toxic activity, this approach may overcome the limitations presented by CT or LT, which limit clinical uses (Holmgren and Czerkinsky 2005; Lycke 2012; Lawson et al. 2012).

Lectins possess the ability to activate the immune system, and this characteristic may also be exploited for oral immunization, since enhanced intestinal absorption by attaching to M cells in PP can be achieved. Plant lectins have demonstrated to be strong mucosal immunogens, stimulating systemic and mucosal antibody responses after oral or intranasal delivery (Lavelle et al. 2000; Rosales-Mendoza and Salazar-González 2014).

The formulation of antigens in various particulate delivery systems for mucosal administration may be advantageous in the following ways: (1) protects the antigen from degradative mucosal enzymes, (2) facilitates the preferential uptake of encapsulated antigen by M cells, (3) sustains the release of antigen to increase the presentation time of antigen to APCs, (4) allows for co-presentation of antigen and adjuvant to APCs, and (5) allows for the induction of cell-mediated immune response by modifying presentation of antigen to APCs. Therefore, rational antigen selection, adjuvants to angle-protective immune responses, efficient vectors to target APCs, and appropriate administration routes are key aspects to take into consideration in the development of efficient mucosal vaccines (Sharma and Hinds 2012; Valiante 2003; Pavot et al. 2012).

Typically, the doses that are required to elicit immune responses by the oral route are substantially higher, by up to 100-fold, than those requiring for parenteral formulations. This raises the crucial issue of the cost of immunization.

Table 2.2 Comprehensive list of adjuvants and delivery vehicles used in mucosal immunization approaches

Adjuvant	Administration route	Antibody	Cellular responses	Proposed mechanism	Soluble factors	Characteristics	Advantages	Disadvantages	References
Cholera toxin	Oral Nasal Vaginal Rectal	IgA, IgG1, IgE	Th2, CD8	Enhancement of antigen presentation by APCs	IL-4, IL-5, IL-6, IL-10	Enterotoxins composed of one active (A) and five binding (B) subunits	Lead to enhanced uptake of Antigens Enhance antigen presentation Promote isotype differentiation in B cells leading to increased IgA production	High toxicity Affinity to central nervous system Increase permeability of the intestinal epithelium	Chadwick et al. 2010; Holmgren et al. 2003; Freytag and Clements 2005; Yamamoto et al. 1997
E. coli heat-labile enterotoxin	Oral Nasal Vaginal Rectal	IgG1, IgG2, IgA	Th1/Th2, CD8	Enhancement of antigen presentation by APCs	IL-8, IL-10, IL-1(α,β), IL-6	ADP-ribosylating activity Binds to GM1 gangliosides expressed on the surface of many cell types			
Cry 1Ac Protoxin	Oral Intranasal intraperitoneal	Preferentially IgG1 IgA IgG2a		Increment of co-stimulatory molecules, CD80 and CD86, on APCs	IL-6 TNF-a MCP-1	Crystal protein 130 kDa From Bacillus thuringiensis	Non-toxic for mammalian Easy and low cost of production Friendly administration Induce mucosal and systemic responses protection against parasite infections	Mechanism of action has not been elucidated completely	Moreno-Fierros et al. 2013; Rojas-Hernández et al 2004
Virus-like particles	Oral Nasal Vaginal	Both serum IgG and IgA	Th1/Th2	Multiple mechanisms	Not determined	Plasmid DNA Proteins Peptides	Lacks viral genes Highly immunogenic High rate of uptake Undergoes self-assembly	Formulated by recombinant technology	Aguilar and Rodríguez 2007:Sharma et al. 2009; Holmgren et al. 2003; Reed et al. 2009; Eriksson and Holmgren 2002; Takamura et al. 2004; Ludwig and Wagner 2007

Table 2.2 (continued)

Adjuvant	Administration route	Antibody	Cellular responses	Proposed mechanism	Soluble factors	Characteristics	Advantages	Disadvantages	References
Protollin (LPS)	Nasal	Serum IgG, IgA	Th1/Th2	TLR4, TLR2	IFN-γ, MIP-3α, IL-18	Inflammatory responses by LPS-binding proteins LBP, CD14, MD-2, and TLR4	Release of inflammatory cytokines and upregulation of costimulatory molecules on antigen presenting cells	Severe toxicity in mammals	Chabot et al. 2005, 2007
CpG	Oral Nasal Vaginal Rectal	IgG2a, IgA	Th1/Th2, CD8	TLR9	IL-6, IL-12, IL-8 RANTES, MIP-1α MIP-1β, TNF-α, IFN-γ	Small oligodeoxynucleotides (ODN) Innate and adaptive immune responses	CpG ODN also directly activates monocytes, macrophages and DCs directly and should be superior adjuvant for intracellular pathogens	Suboptimal in vivo stability Toxicity Unfavorable pharmacokinetic/biodistribution Lack of specificity for target cells Requirement for intracellular uptake	Holmgren et al. 2003; Vajdy et al. 2004; McCluskie et al. 2000
Flagellin	Nasal	IgA, IgG	Th1/Th2	NLRC4, TLR5 and uses only MyD88 as the cytoplasmic adaptor	TNF-α, IFN-γ, MIP-2, IL-6	Flagellin is the only cognate ligand reported so far for TLR5	One of the very limited number of TLR ligand that could be engineered genetically Flagellin is a highly expressed and stable bacterial protein As a mucosal adjuvant, flagellin is almost as potent as CT or LT while it does not accumulate in olfactory nerve and bulb	Must await to determine whether recombinant flagellin-based vaccines trigger adverse events, such as a systemic cytokine storm or intense local inflammation at the site of immunization, which would limit their use	Coffman et al. 2010; Reed et al. 2009; Samatey et al. 2001; Weimer at al. 2009

Table 2.2 (continued)

Adjuvant	Administration route	Antibody	Cellular responses	Proposed mechanism	Soluble factors	Characteristics	Advantages	Disadvantages	References
MPL	Oral Nasal Vaginal Rectal		Th1/Th17	TLR4, TLR2	IL-1, IL-17, IFN-γ	Monophosphoryl lipid A bacterially derived product	Retains much of the immunostimulatory properties of the parent lipopolysaccharide without the inherent toxicity Mediate specific cellular immunity and enhanced levels of complementing antibodies Induce mucosal and systemic responses	Has been used extensively as an adjuvant for parenteral vaccines	Reed et al. 2009; Freytag and Clements 2004
QS21	Oral Nasal	IgG2a, IgG2b, IgG1, IgE	Th1		IL-4, IL-5, IL-6, IL-10	Extracted from the bark of *Quillaja saponaria*	Exhibit a remarkable ability to augment clinically significant responses to vaccine antigens targeting a wide landscape of diseases and degenerative disorders	Semi-purified extract consists of a mixture of triterpene saponins	Reed et al. 2009; Boyaka et al. 2001; Duthie et al. 2011; Chea et al. 2012
Chitosan/chitin	Oral Nasal	Serum IgG, sIgA	Antigen-dependent response	Electrostatic interaction with mucus and cell surfaces	IL-1β, IL-18	Non-toxic, biocompatible, and biodegradable It is converted from chitin by deacetylation	Stimulate macrophages by interacting with receptors Macrophages produce cytokines and other compounds that confer non-specific host resistance against bacterial and viral infections, and anti-tumor activity Chitin is a strong Th1 adjuvant Anti-allergic properties	Limited perception of the importance of the chemical/biochemical characteristics of the isolated chitin or chitosan for the replication of experiments and optimization of results	Reed et al. 2009; Li et al. 2013

Table 2.2 (continued)

Adjuvant	Administration route	Antibody	Cellular responses	Proposed mechanism	Soluble factors	Characteristics	Advantages	Disadvantages	References
Antigen delivery systems									
PLGA microparticles	Oral Nasal Vaginal	Antigen-dependent antibody	Antigen-dependent response	Mechanism undefined	Depends on immunostimulant	PLGA Plasmid DNA Protein Peptide Low-molecular-weight molecules Low loading efficiency	Controlled release Sensitive to environment Stable microenvironment Biocompatible	Degradation of antigen during encapsulation	Aguilar and Rodriguez 2007; Yih and Al-Fandi 2006; Mallapragada and Narasimhan 2008; Sinha and Trehan 2003
4a. PLA						Plasmid DNA Protein Peptide Lipophilic compound	Controlled release Surface easily modified	Low loading efficiency Degradation of antigen during encapsulation Cytotoxicity Reactogenicity	Sharma et al. 2009; Sinha and Trehan 2003; Lassalle and Ferreira 2007; Ogay et al. 2006
4b. PEI Plasmid						Plasmid DNA	Efficiently transfected	Cytotoxicity	Ogay et al. 2006; Forrest et al. 2003; Wong et al. 2006
Emulsions									
Water-in-oil emulsion									Chadwick et al. 2010 Joffret et al. 1990;
Oil-in-water emulsion						Water-insoluble drugs Water-soluble drugs Proteins DNA	Easy surface modification Synthesized from non-toxic material Dual function Wide range of antigen encapsulation	Low antigen loading Low stability	Alving 1991; Aguilar and Rodriguez 2007; Drummond et al. 2000; Sharma et al. 2009; Kersten and Crommelin 2003;
Liposomes						DNA cytotoxic agents Proteins	Efficient endocytic release	Intramembrane repulsion	Yih and Al-Fandi 2006 Drummond et al. 2000; Karmali and Chaudhuri 2007; Yih and Al-Fandi 2006

Table 2.2 (continued)

Adjuvant	Administration route	Antibody	Cellular responses	Proposed mechanism	Soluble factors	Characteristics	Advantages	Disadvantages	References
pH-sensitive liposomes						DNA siRNA	Controlled release of antigen	Non-specific interactions	Sato et al. 2007; Drummond et al. 2000; Drulis-Kawa and Dorotkiewicz-Jach 2010; Karmali and Chaudhuri 2007
Cationic liposomes	Oral Nasal Vaginal	IgG1, IgG2a	Th1/Th2, CD8		Not determined	Composed of antigen, cholesterol, phospholipid and saponins	Antigens evoke protective responses when prepared into ISCOMs	High production cost and low antigen binding	Medzhitov 2001; Reed et al. 2009
ISCOM	Oral Nasal Vaginal	IgG1, IgG2a	Th1/Th2, CD8	Saponin	Not determined	Particulate antigen delivery systems composed of antigen, cholesterol, phospholipid and saponins	Induce strong antigen-specific cellular or humoral immune responses to a broad range of antigens of viral, bacterial, and parasite origin or tumors	High production cost	Medzhitov 2001; Reed et al. 2009; Hong-Xiang et al. 2009
Plants	Oral	IgG IgA	Th1	Gradual release of antigen in the gastrointestinal tract	Depending on the antigen	Plant cells serve as biofactories and delivery vehicles Concept has been proven for a wide variety of human infectious and autoimmune diseases	Low cost Easy storage Absence of mammalian pathogens Delay antigen degradation Possible adjuvant effect mediated by plant metabolites	Variability in expression levels Characterization in each lot of antigen Possible induction of immune response against plant proteins	Govea-Alonso et al. 2013; Yusibov et al. 2011; Kostrzak et al. 2009

Mucus provides a highly viscous and heterogeneous microenvironment that presents a significant barrier not only for pathogen entry but also to mucosal vaccine delivery. Therefore, in order to be effective, mucosal vaccines must prevent inactivation of both the antigen and the adjuvant by the harsh mucosal environment and deliver the vaccine across mucosal barriers to target mucosal tissues and immune cells. The pore size of mucus has been estimated to range 50–1,800 nm. Surface modification of drug-delivery vehicles has proven to be a promising approach to increase both mucoadhesion and mucus penetration. Several natural materials such as chitosan, alginate, and derivatives of cellulose show strong mucoadhesive properties owing to the presence of numerous hydrogen bond-forming groups. The concepts of mucus penetration and mucus adhesion will have a significant role in achieving effective transport of mucosally administered vaccines (Woodrow et al. 2012).

The induction of mucosal immune responses against foreign antigens, microorganisms, and vaccines requires the presence of organized lymphoid tissue, either within the mucosa or in draining lymph nodes. Soluble, non-adherent antigens are taken up at low levels, if at all, and such antigens generally induce tolerance in the intestine. In general, mucosal vaccines are likely to be most effective when they mimic successful mucosal pathogens in the following key respects: They are ideally multimeric and/or particulate, adhere to mucosal surfaces (or even better, adhere selectively to M cells), efficiently stimulate innate responses, and evoke adaptive responses that lead to immunoprotection against the target pathogen (Neutra and Kozlowski 2006). Particulate vaccines have theoretical advantages for mucosal delivery because M cells are known to uptake efficiently microparticles with a diameter of up to 1 μm. Encapsulation of antigens in polymer-based particles can be a promising tool for delivery of vaccines to mucosal sites. However, without proper targeting, these carriers may not be successfully internalized, processed, and presented in a way to direct an immunological response. Targeting APCs, specifically DCs, constitutes another strategy. Most examples of DC-targeting strategies employ the well-characterized DC receptor DC205, DC-specific intracellular adhesion molecule 3-grabbing non-integrin (DC-SIGN), or mannose receptor. Mucosal epithelial cells represent another opportunity for targeting vaccines. Potential targets to address this objective are the epithelial markers FcRn and galactosyl ceramide (Woodrow et al. 2012). Another method that has been employed to favor adhesion between epithelium and the vaccine delivery vehicle consists of using high-affinity targeting ligands against M cells, but only few M cell receptors had been identified. As M cells tend to exhibit unique glycosylation patterns, lectins such as *Ulex europeus* agglutinin 1 (UEA-1), which binds alpha-1-fucose, has been the most widely investigated M cell-targeting molecules in mice (Pavot et al. 2012).

Accordingly, the effectiveness of live pathogens and effective oral vaccines such as the live poliovirus and live attenuated *S. typhi* vaccines is partly a result of their adaptation to survive in luminal environments, due to which they can efficiently invade organized lymphoid tissues in the intestines. Non-living macromolecules, protein-subunit antigens, and non-microbial particles generally evoke weak immune responses when applied mucosally, and thus the use of adjuvants is required in order to alert the mucosa and activate innate signaling pathways in epithelial cells

or in the underlying APCs. However, the major limitation of using live vaccines and adjuvants are associated with toxicity risks (Pavot et al. 2012).

Under this outlook, it is clear that efforts to overcome obstacles in the development of effective mucosal vaccines are mainly directed towards finding more efficient means of delivering appropriate antigens to the mucosal immune system, and towards discovering effective, safe mucosal adjuvants capable of providing protective immunity against infectious agents.

Vaccines based on live attenuated viruses or microbes that have been inactivated by heat or chemicals comprise the majority of licensed vaccines used for the prevention of infectious diseases. To date, these constitute the only vaccines approved for mucosal delivery and the only ones whose efficacy is correlated with effector mucosal immune response (Woodrow et al. 2012). The oral polio vaccine is a live attenuated vaccine that produces serum antibodies as well as local sIgA in the intestinal mucosa, which confers protection from virus entry and multiplication. Other live attenuated vaccines administered via the oral route are licensed for enteric infections such as cholera, typhoid, and rotavirus. The success of live attenuated or inactivated vaccines is attributed to the presentation of multiple immunogens and enhanced second signals that combine and elicit strong antibody responses and long-term memory. However, not all viruses can be attenuated, and the risk of reversion can compromise safety, especially for viruses with ill-defined attenuation. Although inactivation of viruses and bacteria is a more generalized approach and these vaccines are much safer, inactivated vaccines can exhibit loss of antigens or pathogen-associated molecular patterns (PAMPs). This loss results in rapid waning of protective immunity and causes the inactivated vaccines to be less effective than live attenuated vaccines (Woodrow et al. 2012).

Subunit vaccines and conjugated vaccines are a second largest category of licensed prophylactic vaccines. These vaccines are based on pathogen-specific proteins or polysaccharides conjugated to proteins or peptides. Subunit and conjugate vaccines as well as toxoid vaccines are administered primarily by subcutaneous or intramuscular routes and not mucosally. One notable exception is a vaccine against cholera toxin B subunit and the inactivated strain of *V. cholerae* O1. Oral but not parenteral immunization, with inactivated whole-cell cholera bacteria together with cholera toxin B subunit, protects against cholera colonization and toxin binding. This vaccine induces protection-specific mucosal antibodies and provides long-lasting intestinal immunological memory. However, no other examples of successful licensed subunit vaccines that are administered by mucosal immunization and provide protection are available (Woodrow et al. 2012). The use of living microorganism for the delivery of antigens has shown to induce mucosal immune responses at the gastrointestinal and the systemic levels (Neutra and Kozlowski 2006). Therefore, oral delivery of antigens in attenuated bacterial strains is an alternative solution to antigen protection, but raises safety concerns over the delivery vehicles.

Another approach consists on the use of plant-based vaccines, which provide a means to deliver large amounts of a designated antigen in an encapsulated form. Plants have been used to express a wide range of recombinant proteins, including diagnostic proteins, industrial enzymes, and enzymes used in the production

of pharmaceuticals, food additives, therapeutic proteins, antibodies, and vaccine antigens (Daniell et al. 2009). Levels of expression achieved thus far indicate the long-term economic viability of plant-based systems for recombinant protein production. In the case of subunit vaccines, large-scale antigen production in plant systems should be sufficiently inexpensive to allow for delivery of the necessary high dosages anticipated for oral administration. Production of antigens in plant material has the added advantage of encapsulation in the expression host since antigens are naturally encapsulated in the tissue used for recombinant protein production. This encapsulation appears to guard against rapid and complete degradation of orally administered recombinant proteins. Thus, there is the potential for antigen to be gradually released into the gastrointestinal tract as long as plant cells are digested. This should allow for an increased proportion of orally administered antigens to reach effector sites which line the gastrointestinal tract (Pavot et al. 2012; Rosales-Mendoza and Salazar-González 2014). This approach has yielded encouraging results in animals and humans, although the safety of transgenic plans needs to be further evaluated (Mitragotri 2005). Although in its infancy, oral immunization by means of plant-based vaccines augurs a potential source of novel vaccines. Chapter 13 provides relevant "Plant-Based Vaccines as a Global Vaccination Approach: Current Perspectives" perspectives on future research activity that is considered critical to favor the advancement of this technology.

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Chapter 3
Viral Vector-Based Expression Strategies

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Plant Viral Expression Vectors

Since conventional strategies for producing heterologous proteins in plants are limited by low expression levels and a long time is required for generating stable transgenic lines, alternative approaches have been explored to overcome these limitations of the field. Plant viral vectors have been developed to take advantage of the high viral replication rate and the efficient redirection of the biosynthetic resources of the cell to the expression of its proteins. The development of viral vectors over the past few years has lead to highly efficient systems overcoming the limitations of the conventional approaches to a relevant degree. Viral-based expression has been successfully applied using RNA viruses, such as *Tobacco mosaic virus* (TMV), *Cowpea mosaic virus* (CPMV), *Potato virus* X (PVX), *Alfalfa mosaic virus* (AlMV), and *Plum pox virus* (PPV); geminiviruses, which are single-stranded DNA (ssDNA) viruses, have also been recently exploited as versatile and improved viral expression vectors (Hefferon 2012).

In nature, there is a vast quantity of plant viruses showing particular characteristics, such as the organization of genomic material, host range, virulence, and transmission, which are important properties that influence the versatility and convenience of a plant viral vector. Ideally, vectors are designed to reach high-level production under easy genetic manipulation and plant inoculation. The system should also be compatible with feasible purification processes to achieve a low-cost production.

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Two Main Strategies can be Applied in the Design of Viral Vectors

In order to develop a viral expression vector, two main strategies can be applied. One of these systems, designated first generation vectors is based on expressing heterologous proteins in a "full-virus" vector strategy where the coding sequences are under the control of a strong viral promoter, either as an individual antigenic protein or as a fusion protein comprising the CP, and the antigen of interest. In this strategy, the viral vector retains the machinery required for the normal replication cycle, including viral replication, host infection, translation, assembly of mature virions, cell-to-cell movement, movement through the whole plant, reprogramming of the host biosynthetic machinery, and suppression of gene silencing. Under this scenario, several immunogens have been expressed (Gleba et al. 2004). Although the expression levels usually reached under this full virus strategy are considered relevant, reaching up to 10 % of total soluble protein (TSP) of heterologous protein or more than 1 g of recombinant protein per kg of biomass, it is important to note that the efficiency of the first-generation vectors have some limitations, which include the incapability for the production of heterooligomeric proteins and size limitation in the length of protein of interest, as proteins larger than 30 kDa are poorly expressed and, when included in the form of a chimeric CP, epitopes should be 25 amino acids at maximum in length. These limitations encouraged the development of second-generation viral vectors.

On the other hand, rather than using the complete viral genome, second-generation vectors rely on an integrated system with the minimum viral elements required for the vector replication, while some other functions such as DNA delivery are provided by non-viral elements, such as replicon formation via T-DNA delivered by instead delivery *Agrobacterium tumefaciens* (Fig. 3.1). Importantly, these second-generation vectors conveniently avoid the production of functional infectious viral particles; thus, plants do not develop severe infection symptoms aiding the production of the heterologous protein (Gleba et al. 2005). Figures 3.2 and 3.3 show the expression of green fluorescent protein (GFP) in *Nicotiana benthamiana* plants through this approach. Systems based in "deconstructed expression vectors", tipically provide higher yields than those attained by the first-generation vectors, having maximum yields of up to 50 % of TSP or 5 g/kg of biomass in a short period of time, 4–15 days. Importantly, the genes of interest can be larger than those used for first-generation vectors as the vector has been deconstructed; thus, 2-Kb inserts or 80-kDa proteins can be expressed. Besides the virus in which the system is based, the factors influencing the efficiency include the size of the gene of interest, the host plant, and the initial agrobacteria density.

When viral expression systems are applied, protein configuration may comprise one of the following strategies: (1) expression of full-length antigenic proteins that are intended to be used as subunit vaccine without oligomerization. The TMV constitutes a typical example of this kind of tool (Roy et al. 2011); (2) expression of full-length viral antigenic proteins that assemble into Virus Like Particles (VLPs),

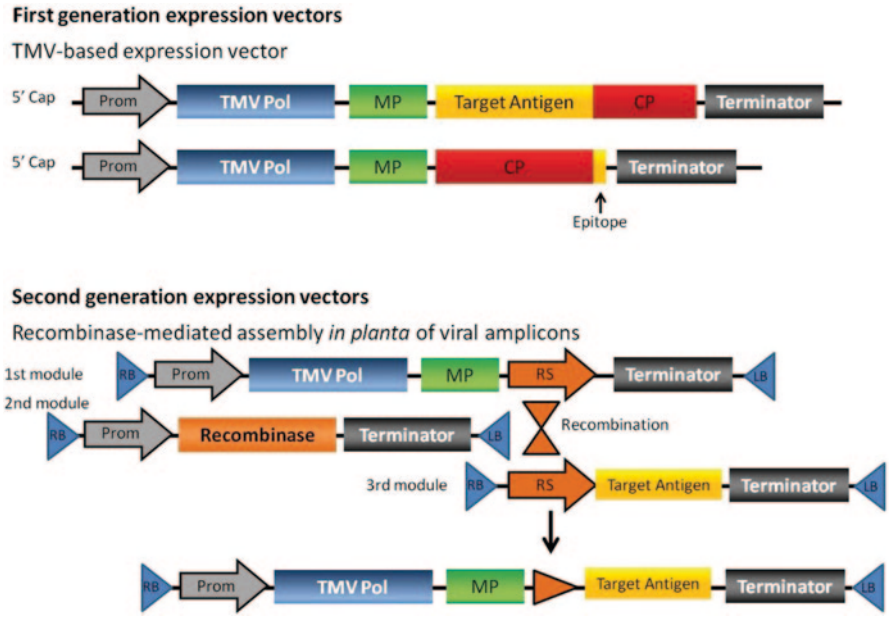

Fig. 3.1 General representation of first- and second-generation vectors. A TMV-based vector containing the endogenous TMV polymerase, movement protein, and a gene of interest fused to the coat protein, or epitopes fused to the C-terminal of CP, under the TMV endogenous promoter and terminator. An example of second-generation vectors relies on the delivery of the viral vector by *Agrobacterium tumefaciens* mediated by the left and right borders. Three separated modules participate in recombination events leading to an *in planta* assembly to form viral amplicons

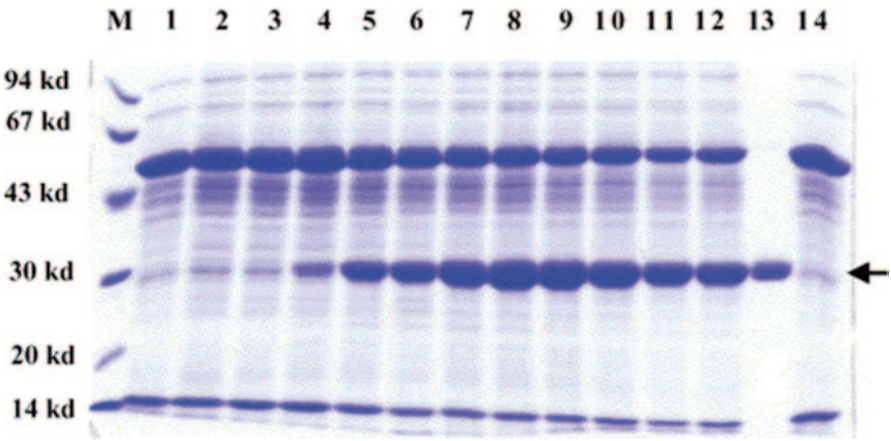

Fig. 3.2 Time course of transgene (GFP) expression in magnifected leaves of *Nicotiana benthamiana*. Coomassie-stained polyacrylamide protein gel showing: *M*, protein standards in kDa; *1–14* are crude extract from uninfected leaf tissue; *2–12* are crude extract from infected leaf tissue, 2–12 days after infiltration; *13* GFP standard; the *arrow* indicates the GFP. (Taken from Gleba et al. 2005)

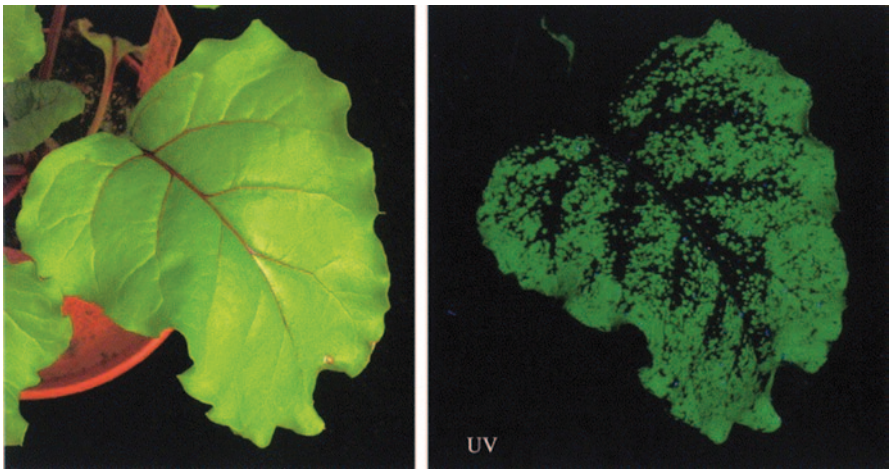

Fig. 3.3 Leaf of edible plant (red beet) expressing GFP, 7 days after infiltration; the picture on the *right* is photographed under UV light. (Taken from Gleba et al. 2005)

which resemble a virus but lack of genetic material; (3) production of chimeric VLPs (CVLPs) through a viral expression system that retain the production of an envelope or nucleocapsid protein that are engineered to display heterologous epitopes through genetic fusions (Ludwig and Wagner 2007, Fig. 3.4); (4) production of chimeric viral particles (CVP) through a viral expression system based in the production of replicative viruses engineered to display heterologous epitopes through genetic fusions to the coat protein. This approach has been extensively applied to the CPMV (Sainsbury et al. 2009). The last two configurations should take into consideration the size and the amino acid composition of the target epitope to avoid major structural changes that may prevent CVP or VLP assembling. Epitopes can be fused at the amino or carboxyl terminal region of the coat protein (CP) of these viruses or at specific sites into the protein where the epitope is exposed on the particle surface without abolishing its assembling (Santi et al. 2006a).

RNA Viruses Used as Expression Vectors

The most notable advancement in the field of plant virus vectors is derived from RNA viruses, and their use for vaccine production and biopharmaceutical proteins has been exploited during the past two decades (Yusibov et al. 2011). Among the deconstructed expression vectors derived from the RNA viruses that have successfully been used to produce a variety of plant-made vaccines are: TMV, CPMV, PVX, and PPV (Lomonossoff and Hamilton 1999; Cañizares et al. 2005; Chung et al. 2011), which are analyzed in the following subheadings.

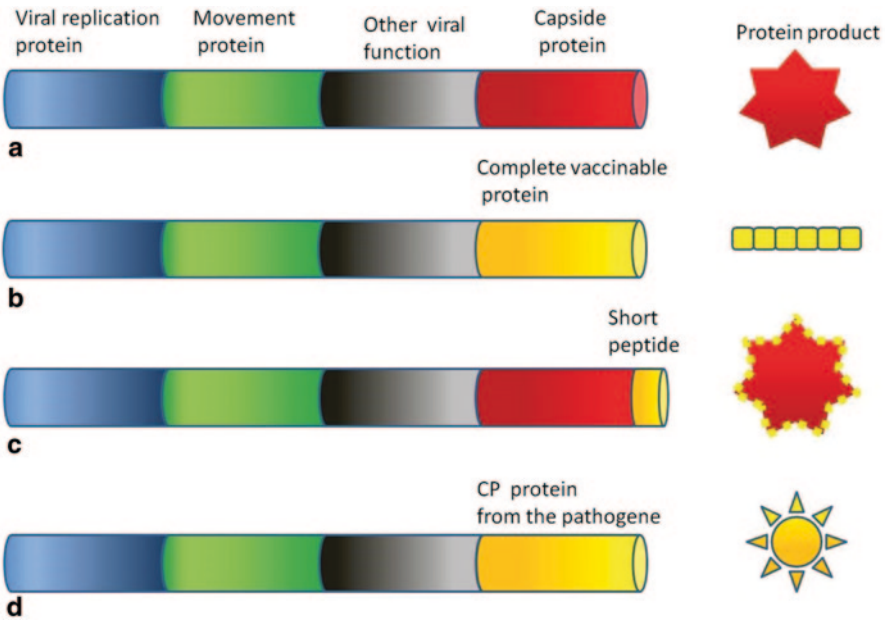

Fig. 3.4 Distinct types of antigen configurations for vaccines production using plant viral vectors. **a** Genomic organization of wild-type plant virus. **b** Substitution of CP protein by the target antigen (protein or peptide). **c** Addition of a short peptide to CP protein to obtain chimeric virus particles. **d** Substitution of native CP gene by the CP or the envelop protein of the target pathogen

TMV-, PVX-, and PPV-Based Vectors

These viruses are rod shaped with their N-terminal or C-terminal CPs exposed at the virion surface and theoretically can overcome packaging constraints imposed by spherical viruses, which implies that they could be more flexible and stable for expression of foreign genes making them an ideal epitope presentation system. TMV is the most utilized plant virus as expression vector for biopharmaceutical protein production. TMV has a single-stranded plus-sense RNA virus encapsidated by 2,130 molecules of CP in which small peptides can be fused at the genetic level. Epitope length to be incorporated onto the virus particle surface is under strict limitations. Examples of some of the epitopes that have been expressed using TMV vector include the Human papillomavirus (HPV), *foot-and-mouth disease virus* (FMDV), *Human Immunodeficiency Virus* type I (HIV-1), malaria, and rotavirus, among others (Turpen et al. 1995; Wigdorovitz et al. 1999; Noris et al. 2011). Full-length proteins have been also expressed by means of TMV expression vectors.

One variant consists of the expression of both the endogenous CP and the modified CP under the control of an additional CP subgenomic promoter in the viral genome (Fig. 3.5). Examples of full-length immunogenic proteins expressed from TMV-based vectors include L1 and E7 proteins of HPV, VLPs based on the CP of *Norwalk virus,* MSP4/5 *Plasmodium* antigen, α-trichosanthin, *bovine herpes virus*

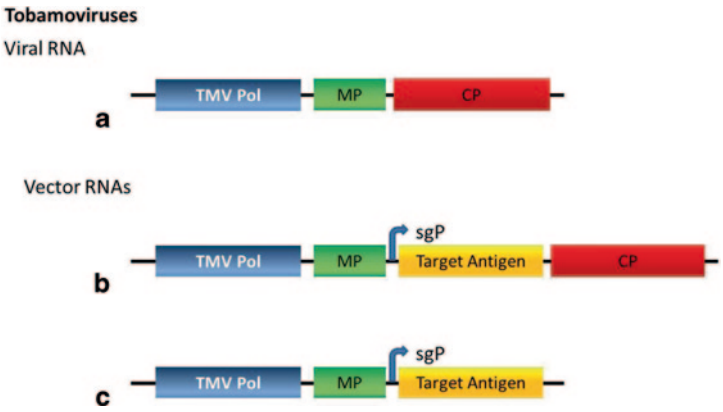

Fig. 3.5 General representation of *Tobamovirus*-based vectors. **a** Genomic organization of *Tobamovirus*; the single-strand RNA encoded 3 proteins, the polymerase protein for replication, movement protein (*MP*), and capsid protein (*CP*). MP and CP have a subgenomic promoter (sgP). **b** Duplication of CP promoter (sgP) in order to co-express the gene of interest along with the CP protein; the gene of interest (target antigen) is cloned under a subgenomic CP promoter. **c** Substitution of the CP protein by the target antigen

gD-protein, major antigen birch pollen, among others (Kumagai et al. 1993; Pérez Filgueira et al. 2003; Wagner et al. 2004; Gils et al. 2005; Santi et al. 2008; Webster et al. 2009; Noris et al. 2011).

TMV has been also used as a "deconstructed" expression vector where the rationale consists of separating the genome into a number of modules, which contain the minimal sequences for replication and cassettes designed for the insertion of foreign genes. These modules of the recombinant virus vector are flanked by the left and right borders from *A. tumefaciens*; thus, they can be then transferred into plants using the T-DNA transfer mechanism of the agrobacteria. This technology known as "magnifection" was established by Icon Genetics in 2005. In this approach, a suspension of *A. tumefaciens* strains carrying the distinct modules are infiltrated under the aid of vacuum into the intercellular spaces of the mature plant leaves, resulting in a rapid and synchronous systemic infection and the protein can be obtained around 1 week post infiltration (Gleba et al. 2004).

The fundament of this innovative system has been used by many researchers and industry. For example, Lindbo (2007) used a TMV vector in conjunction with a viral RNA silencing suppressor to produce high levels of recombinant protein (from 600 to 1,200 mg of recombinant protein per gram of infiltrated tissue) within 1 week post-infection. Another group produced a vaccine against *Yersinia pestis,* the causal agent of plague. The plant-produced plague antigens V, F1, and F1-V were purified from this system and elicited systemic immune responses and provided protection against aerosol challenge by virulent *Y. pestis* in guinea pigs, reflecting the immunoprotective potential of the vaccine (Santi et al. 2006b).

The research group of Saejung et al. (2007) was also able to use a TMV-based deconstructed transient expression system to express the envelope protein of the *Dengue virus*. Mice immunized intramuscularly with plant-derived dengue envelope protein D2EIII showed neutralizing activity against the type 2 dengue virus (Saejung et al. 2007). As another example, production levels up to 300 mg/kg of fresh leaf weight were determined for the *Hepatitis B virus* surface antigen (HBsAg) expressed in magnICON®-based TMV viral expression vector system (Huang et al. 2008). These hepatitis B virus (HBV) vaccine antigens had the added advantage of being able to self-assemble into VLPs, which is relevant in terms of immunogenicity. Finally, Webster et al. (2009) expressed the malarial antigen PyMSP4/5 in *N. benthamiana* leaves using the same TMV deconstructed vector described above. Malarial antigen expressed reached levels up to 10% of the TSP or 1–2 mg/g of fresh weight. Furthermore, the antigens retained their immunogenicity after long-term storage at room temperature in the freeze-dried leaves. Mice that were fed this malaria plant-derived antigen along with a mucosal adjuvant produced antibodies specific to malaria, supporting the concept that large quantities of vaccine against malaria can be produced and stored using this TMV-based production system.

A novel design used for the production of vaccines has been developed by Musiychuk et al. (2007) consisting of the pBI121 *Agrobacterium* binary vector containing the TMV genome. Using this expression system, multiple copies of ssDNA sequences are delivered which enhance the viral replication in the plant host, leading to yields of about 100 mg of protein per kg of plant tissue in less than 1 week. Vaccines produced with this platform include the release oncogenic HPV E7 protein, HA, and NA proteins of the H5N1 influenza virus, and V and F1 antigens of *Y. pestis*. Importantly, these plant-derived vaccines have showed to successfully elicit protective immunity in animal models (Gopinath et al. 2000; Massa et al. 2007; Mett et al. 2008).

PVX belongs to the genus *Potexvirus* and is constituted by approximately 1,300 CP subunits encapsidating a single plus-sense RNA molecule with a 5′ endcap and a 3′ poly A tail. The genome encodes for five open reading frames (ORFs). The first ORF encodes the RNA-dependent RNA polymerase required for replication, a set of three movement proteins known as the triple gene block (TGB), and the CP which is required for virion assembly, cell-to-cell infection via plasmodesmata, and systemic movement. In plants, PVX has been utilized as a full-length expression vector capable of infecting distal tissues as well as a deleted vector lacking viral genes essential for local and systemic movement.

Several strategies to develop transient expression vectors derived from PVX have been explored. These strategies include (1) the use of a duplicated sub-genomic promoter to drive exogenous gene expression (Baulcombe et al. 1995), (2) the expression of foreign proteins fused directly to the N-terminus of a truncated CP gene (Marusic et al. 2001; Uhde et al. 2005), (3) the expression of CP and the antigen of interest by means of a single transcription unit but including the FMDV 2A catalytic peptide between both ORFs (Santa Cruz et al. 1996), and (4) the expression driven by a CP sub-genomic promoter of a bicistronic mRNA containing both the foreign gene and CP gene, separated by an internal ribosome binding site (Toth et al. 2001; Fig. 3.6).

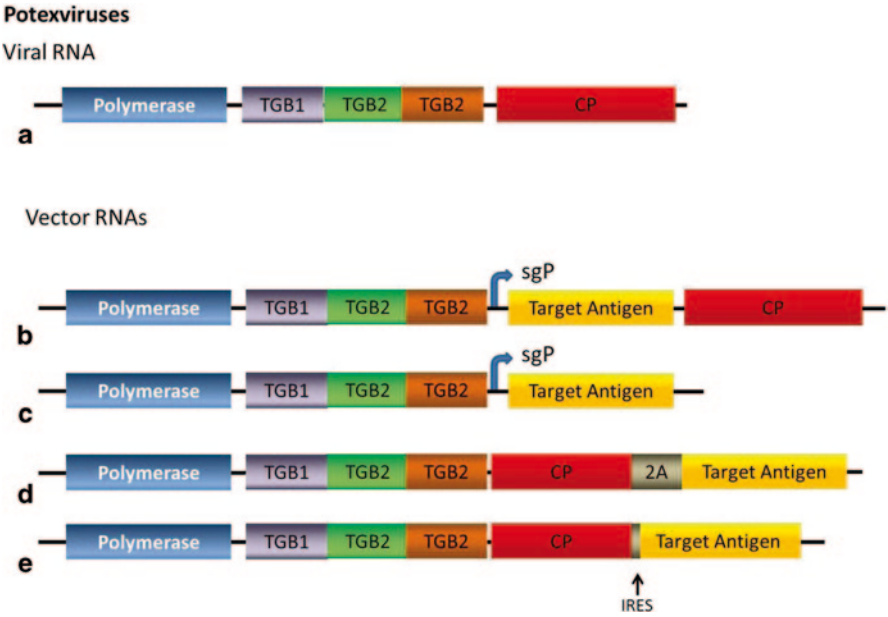

Fig. 3.6 Viral vectors design based on *Potexvirus*. **a** Genomic organization of potexvirus. **b** Duplication of CP promoter to drive exogenous gene expression. **c** Expression of foreign proteins fused directly to the N-terminus of a truncated *CP* gene. **d** Expression of CP and the target antigen in the context of self-cleaving polyprotein by a "ribosome skipping" mechanism mediated by the viral 2A catalytic FMDV peptide. **e** Expression of CP and target antigen in a bicistronic arrangement, comprising an internal ribosome binding site (IRES)

One of the first promising studies in this field was the use of a PVX-derived expression vector called pGR106, constructed by Čeřovská et al. (2008) which was used to express the epitopes from both E7 and L2 proteins of HPV in *N. benthamiana*. The yield obtained was 2.8–4.3 mg/100 g of fresh leaves. Another effort made by Marusic and coworkers aimed to produce vaccines using this system by evaluating the production of CVPs comprising the ELDKWA epitope from gp41 of HIV-1 fused to the N-terminal of CP. Immunogenicity evaluations conducted in mice revealed that mice immunized intraperitoneally or intranasally elicited high levels of HIV-1-specific immunoglobulin G (IgG) and IgA antibodies even when administered in the absence of adjuvants (Marusic et al. 2001).

Recently, Čeřovská et al. (2012) have also reported the production of a fusion protein carrying an epitope derived from HPV-16 L2 minor capsid protein to the PVX CP, achieving production levels of 170 mg/kg of fresh leaf tissue. Mice immunization experiments showed that this candidate is able to elicit antibodies against PVX CP and the L2 epitope when subjected to subcutaneous injection or tattoo administration.

Two main strategies to produce foreign proteins in plants with PPV-derived vectors have been reported. The first consists of an antigen presentation system where the epitopes can be fused to over the 2,000 copies of CP. Under this scheme,

chimeric PPV expressing a single (15 aa) or a tandem repeat (30 aa) of the 6L15 antigenic peptide of *canine parvovirus* (CPV) VP2 protein were recognized by anti-CPV antibodies and elicited high levels of neutralizing antibodies in mice and rabbits (Casal et al. 1995). Another strategy on the PPV-based viral vector requires the insertion of the foreign sequence into the single ORF that encodes a multifunctional polyprotein between the polymerase (Pol) and CP genes, which is self-processed by proteinase domains, resulting in mature viral proteins via proteolysis (Guo et al. 1998). This focus was achieved by flanking the inserted sequence with the proteinase recognition signals. Fernández-Fernández and coworkers used a PPV vector to express the VP60 structural protein from rabbit hemorrhagic disease virus (RHDV) and, importantly, the immunized rabbits were protected against subsequent challenges with a lethal dose of RHDV (Fernández-Fernández et al. 2001, 2002).

Alfalfa mosaic virus (AlMV) has been used for targeting the expression of a 21-amino-acid peptide of the respiratory syncytial virus (RSV) G protein, fused to the AlMV CP, resulting in chimeric particles able to elicit strong T cell responses in humans and both T cell and B cell responses in non-human primates (Yusibov et al. 2005).

CPMV-Based Vectors

CPMV has a bipartite genome, consisting of two molecules of positive-strand RNA: RNA1 and RNA2, which are efficiently replicated in host plants and can easily incorporate vaccine epitopes on its exposed loops of the icosahedral virion surface and can also be simply purified from plants. When designing CPMV-based vectors, the RNA1 component usually remains unchanged, as it contains the genes essential for replication; this allows most of the ORF RNA2 to be eliminated without affecting the ability of RNA2 replication. On the other hand, RNA2 can be approached under two different strategies. One of these approaches involves the insertion of foreign genes into RNA2 genome as the other viral expression strategies where the increase in the size does not have a deleterious effect on viral systemic dissemination. This strategy is relevant for the production of VLPs. The second one, which is frequently more efficient, allows for the insertion of larger foreign genes as a framework at the C-terminal fusion of the polyprotein encoded by the RNA2 molecule, but suppressing the ability of the virus spread within and between the plants (Sainsbury et al. 2009). Under these scenarios, the recovery of the target protein can be performed using a proteolytic inducible cleavage upstream of the foreign protein sequence (Gopinath et al. 2000). Unfortunately, this vector system lacks natural suppressor (Liu et al. 2004); thus, another component (e.g., the HC-Pro potato virus Y) must be supplied. This is usually accomplished through agroinfiltration, which allows the simultaneous delivery of multiple gene expression constructs into plant cells. The use of "deleted" RNA2 variants offers a higher yield of the target protein and is clearly advantageous in the case of antibody production or heterooligomeric antigens (Sainsbury et al. 2008). Moreover, simplified strategies have been designed, consisting of CPMV and expression cassettes encoding the

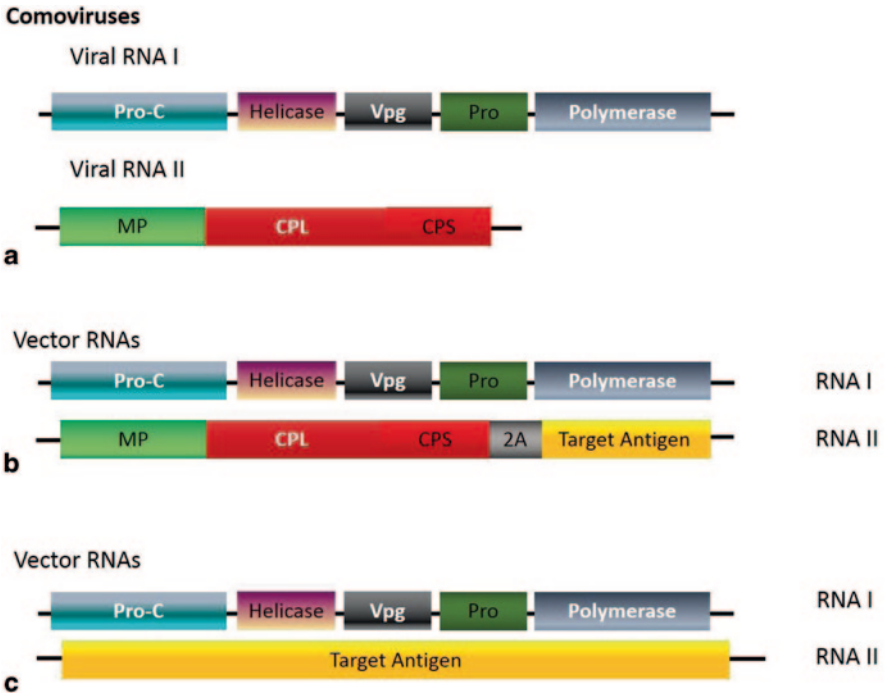

Fig. 3.7 Genomic organization of *Comovirus* and viral vector design. **a** Genomic organization of *Comovirus*. *Pro-C* proteinase cofactor, *VPg* viral protein genome, *Pro* proteinase, *MP* Movement protein, *CPL* Large Coat protein, *CPS* Small Coat Protein, 2A foot-and-mouth disease virus (FMDV) 2A self-cleaving peptide, GEN I Gen of interest. **b** Viral vector obtained by fusing the sequence of the peptide 2A and the gene of interest at the 3′ of the CPS protein. **c** Elimination of the genes *MP*, *CPL*, and *CPS* in RNA2 for viral vector design. The vector maintains the 5′ and 3′ elements required for replication and the gene of interest substitutes MP, CPL, or CPs

PVX P19 sequence incorporated into the T-DNA region of binary vectors, resulting in high levels of expression for multiple polypeptides when a single construct is infiltrated (Sainsbury et al. 2009; Fig. 3.7).

DNA Viruses Used as Expression Vectors

Geminiviruses

Geminiviruses, named for their twinned capsid morphology that encapsidates ssDNA, are a family of plant viruses with broad host ranges, which can replicate at high copy number in infected cells. Monopartite geminiviruses, such as maize streak virus (MSV), contain a single genomic DNA, whereas bipartite geminiviruses, such as *Tomato golden mosaic virus* (TGMV), have a segmented genome (Fauquet et al. 2008). More recently, geminiviruses have been engineered for the production of

plant-made biopharmaceuticals. *Bean yellow dwarf virus* (BeYDV), a member of the mastreviruses, has been modified so that its replication initiator protein (Rep), which mediates virus replication, is under independent promoter control. Using this approach, BeYDV-based expression vectors have been used for producing a vaccine against staphylococcal enterotoxin B (SEB), considered a potential biowarfare agent. In addition, HBV, Norwalk virus, HIV, and HPV antigens have been also produced using this method. The BeYDV expression system has also been used to produce monoclonal antibodies against Ebola virus (Huang et al. 2010).

Another *Geminivirus*, known as *Beet curly top virus* (BCTV), has been engineered in a similar manner to obtain a deconstructed viral vector. Researchers working with this deconstructed virus have demonstrated that foreign protein expression could be, in addition, enhanced via co-delivery of an additional plasmid which expresses a viral suppressor of RNA silencing (Kim et al. 2007).

In general, the strategy using *Geminivirus* as viral vectors consists of flanking the expression cassette by two viral replication origins that are recognized by the viral Rep protein. In plants, once produced, the Rep protein can replicate the DNA flanked by ori sequences. This mechanism is represented in Fig. 3.8.

Vaccine Production in Plants Using the VLP Approach

Hepatitis B Virus

In 1992, Mason and coworkers were the first in demonstrating that a human virus envelopment protein is capable of forming VLPs. In this study, the surface HBsAg was expressed in tobacco plants. As shown in Fig. 3.9, VLP is a particle formed by the capsid protein but without containing genomic material of the virus (Mason et al. 1992).

Subsequently, Thanavala in 1995 and Huang in 2005 demonstrated that HBsAg VLPs produced in plants are able to induce a potent B cell and T cell immune response in mice when administered by the parental route (Thanavala et al. 1995; Huang et al. 2005). These studies open the path of exploring the VLP production in plants, and were followed by several developments on distinct pathogenic viruses.

Human Papillomavirus

Researchers in the Czech Republic have established a system where a 12-amino-acid epitope from L1 protein (aa 108–120) of HPV-16 was fused to either N- or C-terminus of the CP of PVX. The N-terminal fusion was expressed at 173 mg/kg of fresh leaf tissue and the VLPs were immunogenic in mice. This N-terminal fusion showed to result in better yields than C-terminal fusion. The movement of the construction is aided by a TMV MP stably expressed by the transgenic *N. benthamiana* used as expression host. The infected leaves obtained from the first inoculated plant

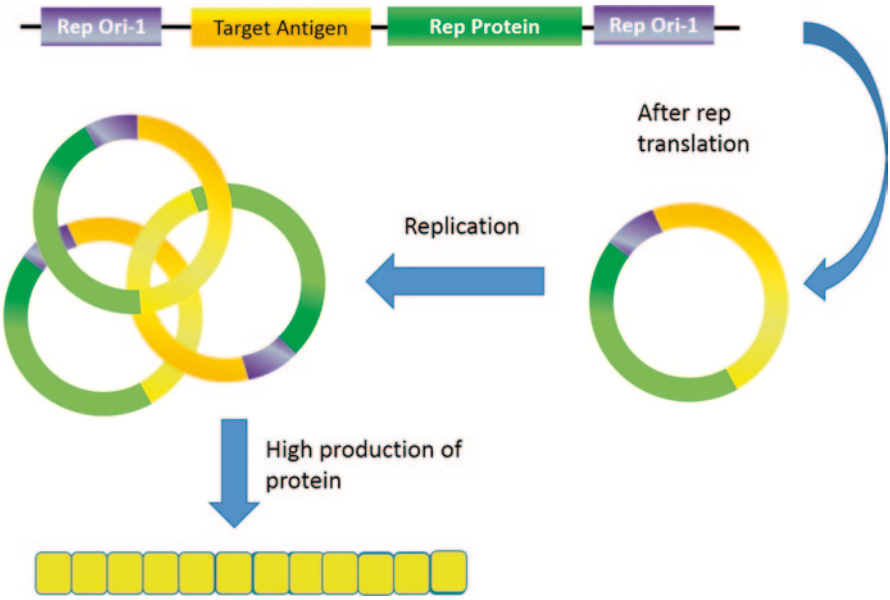

Fig. 3.8 Representation of the *Geminivirus*-based expression strategy. The target antigen gene along with the *Geminivirus* Rep gene is flanked by the viral replication origin (hemidimer arrangement). Rep protein is produced in plant cells, acting over Ori 1 and Ori 2 releasing and then replicating a circular ssDNA viral genome. The high copy number of viral genome allows for the production of high levels of protein

were able to infect new plants by mechanical inoculation after 3 days. This is an advantage for production; however, biosafety aspects should be considered as full containment is required to avoid viral spread to non-target crops (Čeřovská et al. 2012).

The protein L1 of VPH 8 was produced in a non-replicating CPMV-derived expression vector. The C-terminus truncated version of L1 accumulates at higher levels than the complete L1 protein, which may be due to the absence of nuclear localization in the truncated version CPΔ22. Truncated L1 was obtained at levels of 240 mg/kg of fresh leaf material. The production level is compared with chloroplast system for L1 of other VPH (137 mg/kg). The truncated protein maintained the ability to assemble in VLPs (Matic et al. 2012).

Influenza Virus

For influenza, the co-expressions of HA and M1 were first used to form VLPs, but expression of HA alone is also able to yield VLPs.

VLPs based on the H5 protein from the Indonesia H5N1 strain were produced in plants, and purified and evaluated in a phase I clinical trial. This vaccine showed acceptable immunogenicity in humans and did not produce serious side effects (Landry et al. 2010).

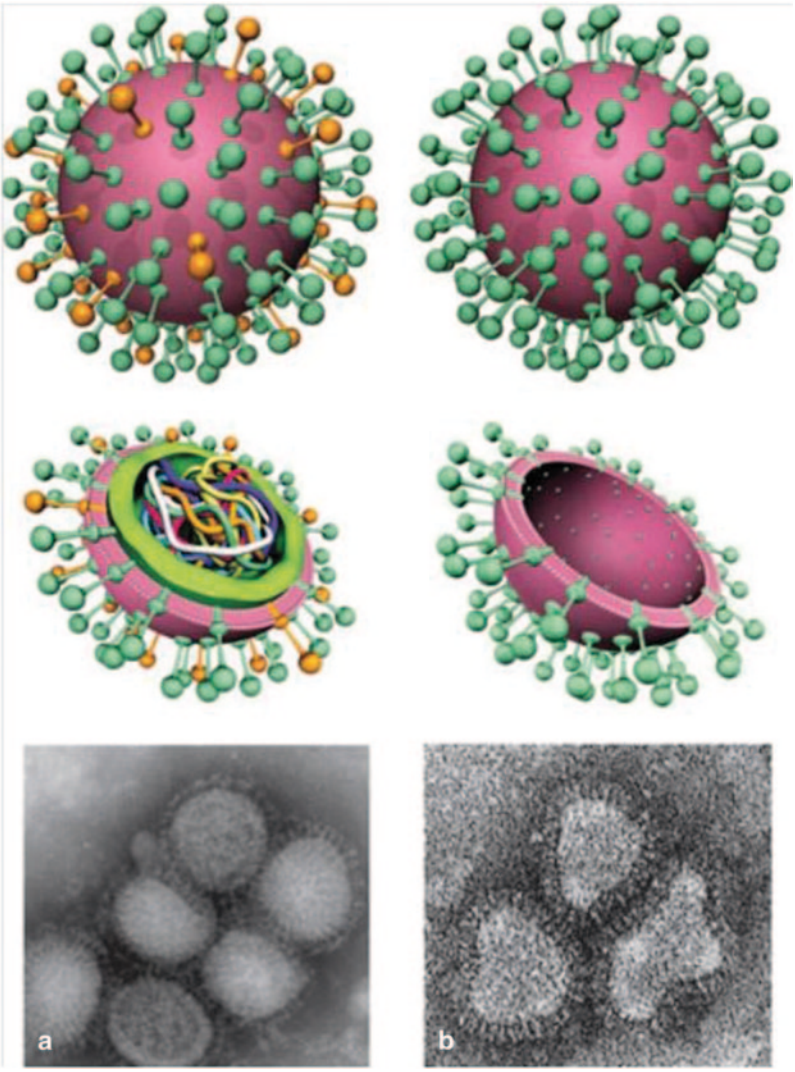

Fig. 3.9 Comparison between influenza viral particles (**a**) and virus-like particles produced in plants (**b**). (Taken from D'Aoust et al. 2010)

Chimeric proteins expressing influenza epitopes in a different VLP backbone have also been developed. In particular, L1 protein of VPH 16 was used to design chimeric VLP (CVLP) displaying the M2e influenza epitope. The CVLP was expressed in *N. benthamiana* through a CPMV. L1 was deleted at the carboxyl-terminal region and the epitopes were located in an internal L1 region. The chimeric L1 was able to form VLPs exposing the M2e peptide. The system produced up to 120 mg of recombinant protein per kilogram of fresh tissue (Matic et al. 2011).

The epitope of the conserved transmembrane domain of M2 of the influenza virus was fused to hepatitis B core antigen (HBc) and expressed by the PVX in *N. benthamiana.* Levels of 2 % of TSP were obtained. The purified protein was immunogenic in mice and the response was Th1 polarized (Ravin et al. 2012).

Chapter 7 presents a detailed analysis on the advanced models of plant-based influenza vaccines.

Human Immunodeficiency Virus

The *Human immunodeficiency virus* (HIV) has been also explored as a target of plant-based vaccines. Some of these approaches are based on VLPs. Greco et al. 2007 showed that epitopes of HIV fused to HBsAg can assemble into VLPs when produced in transgenic tobacco and *Arabidopsis*. Viral vectors also have been used to transient expression of HIV VLPs. Meyers et al. (2008) used a TMV vector to express the Pr55Gag protein in *N. benthamina*. VLPs obtained in this system were similar to those produced in insect cells and also induced a Gag-specific antibody response in mice after three intraperitoneal injections. The other way to obtain a vaccine has been to design chimerical VLPs with plant virus. McLain et al. (1995) fused a peptide of 22 amino acids from the gp41 envelope protein of HIV-1 strainIIIB (amino acids 731–752) to the plant viral vector CPMV. Chimeric VLPs were demonstrated to induce gp41-specific neutralizing serum antibodies in mice when immunized subcutaneously via and in the presence of alum adjuvant. In addition, mice vaccinated with these VLPs in the presence of cholera toxin mucosal adjuvant via intranasal administration developed both mucosal and systemic HIV-1-specific IgA and IgG2a antibody responses. Marusic in 2001 used a highly conservative ELDKWA epitope from gp41 fused to the N-terminus of PVX CP, and the purified chimeric VLPs obtained induced high titers of neutralizing HIV-1-specific IgG and IgA antibodies in mice when IP or IN delivery was done. Another subunit vaccine has used the V3 loop from HIV-1 gp120 fused to AlMV CP and expressed by TMV-based vector. *N. benthamiana* inoculated with the vector produced chimeric viral particles which were shown to elicit antigen-specific virus-neutralizing antibodies in intraperitoneally-immunized mice.

Norwalk Virus

Norwalk virus has been also used to form VLPs in tobacco and *N. benthamiana* (Mason et al. 1996). These VLPs have showed to be immunogenic and safe in a phase I clinical trial, where a group of 20 volunteers were immunized with the plant-based formulation (Tacket et al. 2000). Recently, Lai et al. (2012) produced VLPs of NWV at high levels in lettuce plants using a geminiviral vector (Fig. 3.10). Using lettuce plants, the purification process of proteins is less tedious because of the absence of phenolic compounds in lettuce than the common plants for protein expression as *N. tabacum.*

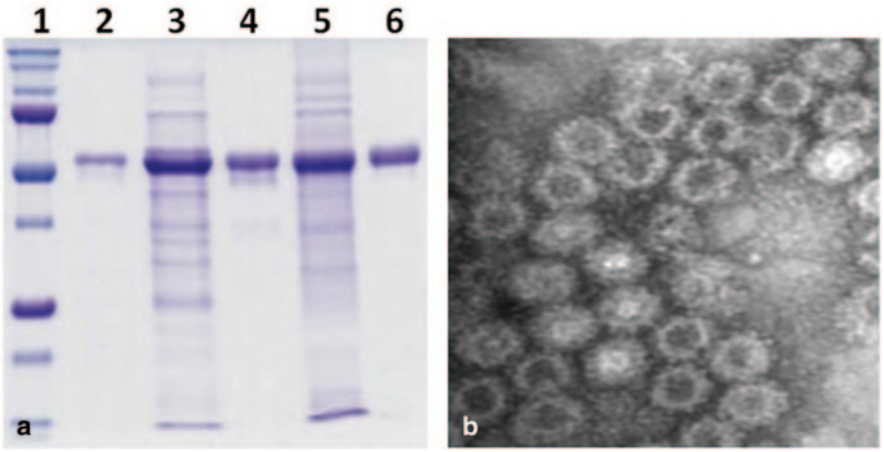

Fig. 3.10 **a** Production of *Norwalk virus* capsid protein (NVCP) by a geminiviral vector in *N. benthamina* and lettuce plants (*lane 1*: molecular weight marker; *lane 2*: insect cell-derived NVCP reference standard; *lanes 3 and 4*: crude protein extract and purified NVCP from *N. benthamiana* leaves as a comparison; *lane 5*: crude extract from pBYNVCP/pREP110 infiltrated lettuce leaves; *lane 6*: purified NVCP from lettuce leaves). **b** Characterization of VLP formation. (Taken from Lai et al. 2012)

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Chapter 4
Plastid-Based Expression Strategies

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Introduction

Worldwide need for new vaccines has led to the development of new production platforms. Plant-based vaccines are a promising platform that is augured as an alternative that is able to address some limitations in the field, comprising high costs and low coverage. Nuclear transformation strategies allow for the insertion of the transgene in the complex nuclear genome, leading in general to low yields (see Chap. 1); therefore, the demand for high-yield platforms has led to alternative approaches to override this limitation.

Early procedures on plant genetic transformation were carried out at the nuclear level using *Agrobacterium tumefaciens* as transformation vector since this pathogen mediates the integration of specific DNA sequences into the nuclear genome. Although these systems were efficient in several plant species, monocots, including the main cereal crops (wheat, rice and maize), could not be efficiently transformed by *A. tumefaciens* (DeCleene and DeLey 1976) in the early stages of the transformation protocol development. In addition, approaches requiring a high level of expression are hampered by the low yields frequently observed under nuclear expression.

Plastids are plant organelles derived from endosymbiosis of a common ancestor of the present cyanobacteria (Martin et al. 2002). In plant cells, they participate in a wide range of metabolic processes (Leister 2003). The chloroplast, the best known among plastids, is the site of photosynthesis and an important number of

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biosynthetic pathways, including those for fatty acids, amino acids, isoprenoids, etc. All plastids derive, directly or indirectly, from a small and undifferentiated organelle termed the proplastid and each plastid or proplastid has a number of small circular DNA molecules (plastome) of about 120–160 kb.

Chloroplast-based expression was proposed for biotechnological purposes in the early 1990s. A plant cell contains approximately 100 chloroplasts and each chloroplast at the same time has a high number of copies of their self-replicating DNA (approximately 100 copies). This implies that a single gene is represented by at least 10,000 copies in a single leaf plant cell. In addition, it should be considered that chloroplast DNA possesses two inverted repeat regions and thus the copy number of the genes encoded by this region is doubled when transgene insertion takes place in that region. This factor, in addition to a general absence of silencing phenomenons, favored high transcription rates and thus can achieve very high expression rates for heterologous proteins (Maliga 1993; Wakasugi et al. 2001). In tobacco, this technology has allowed for obtaining yields of up to 70 % of the plant's total soluble protein (% TSP) (Oey et al. 2009).

On the other hand, biosafety occupies a critical place in the regulatory aspects of biotechnology applications. In this sense, genetically modified organisms (GMOs) have been under the spotlight. The transgene leakage and transversal gene transfer to other species is a central issue. In this context, it deserves a special consideration that in most plant species plastids show uniparental maternal inheritance (Hagemann 2004), which minimizes the risk of undesired gene flow via pollen. For example, maternal inheritance and prevention of gene flow via pollen has been shown in tobacco and tomato plants (Daniell et al. 1998; Ruf et al. 2001). Some recent studies showed that there is a very low frequency of paternal transmission of plastids in tobacco (Ruf et al. 2007; Svab and Maliga 2007). Although transgenic plants can be grown under controlled conditions to assure gene containment, chloroplast transformation is a competitive system that provides an opportunity of growing transplastomic plants in the fields with no risk of undesired gene flow via pollen.

Since transfer of heterologous DNA to chloroplasts by means of *A. tumefaciens* is not successfully accomplished, a number of alternative transformation approaches have been developed, including physical methods for the DNA transfer, being particle bombarment and the polyethylene glycol (PEG)-mediated method the most used approaches, which allows for an efficient transformation of plastid genomes (Maliga 1993; Maliga et al. 1993; Koop et al. 1996).

The first report on stable plastid transformation in higher plants was published by Maliga's group in the early 1990s. The integration of transgenes into the chloroplast genome differs from the non-homologous random recombination of the nuclear transformation mediated by *A. tumefaciens*. Chloroplast genome transformation is typically achieved by site-specific homologous recombination (Svab et al. 1990; Staub and Maliga 1992; Svab and Maliga 1993; Maliga 1993; Daniell 2002; Daniell and Dhingra 2002). This is accomplished by designing specific chloroplast transformation vectors that carry homologous sequences flanking the transgene expression cassette. Specific chloroplast transformation vectors are required in this methodology. Using this approach, several insertion sites have been targeted thus far (Verma et al. 2008). Vector design aspects will be elaborated on in a further section of this chapter.

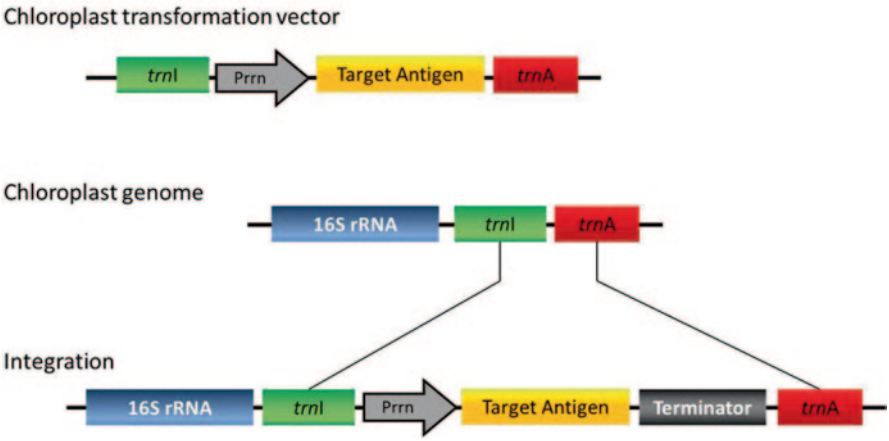


Fig. 4.1 General representation of integration of foreign genes into chloroplast genome. The chloroplast transformation vector contains the *trn*A and *trn*I genes from the inverted repeat (*IR*) region of the tobacco chloroplast genome as flanking sequences for homologous recombination, and the target antigen is under the 16 ribosomal RNA gene promoter (*Prrn*)

Advantages of using chloroplast-based expression for the production of plant-based vaccines can be summarized as follows:

1. Transgene confinement is offered by this approach because of the maternal inheritance shown by most plant species (Daniell 2007; Ruf et al. 2007).
2. High yields can be achieved due to the high copy number of the plastome and chloroplasts per cell as well as a typical absence of silencing events and position effects due to site-directed insertion (Maliga 2002; Koop et al. 2007; Bock and Warzecha 2010).
3. Simultaneous expression of a number of genes in operon-like arrangements is possible, which would allow for the production of multicomponent vaccines (Arai et al. 2004; Nakashita et al. 2001).

This chapter covers the principles of chloroplast genetic engineering and the most advanced approaches developed with this technology as well as a prospective view of the field.

Principles of Chloroplast Expression Systems and Expression Vector Design

Chloroplast genetic engineering relies on using transformation vectors targeting specific sites at the genome by means of homologous recombination. A basic chloroplast-specific expression cassette comprises a promoter, the gene of interest, a selection marker, and 5′/3′ regulatory sequences to enhance the efficiency of transcription and translation (Fig. 4.1). All of these elements are flanked by

chloroplast-homologous sequences that mediate site-specific integration. *Peste des petits ruminants virus* (pPRV) vector series are representative of this basic configuration and are considered the first-generation plastid vectors (Zoubenko et al. 1994).

The integration site at the chloroplast genome is defined by the objective of the transformation. Plasmids targeting the inverted repeated region (IR) typically achieve higher expression levels since two copies of the transgene are present per chloroplast genome; thus, this kind of vectors is preferred for the production of plant-derived vaccines as high yields of the antigenic proteins are required. In contrast, plasmids targeting genome locations outside of the IR lead to a single transgene copy, events that in general result in relatively lower protein yields and are rather used when the objective is directed to studying some plastid translational or transcriptional mechanisms (Bally et al. 2009).

Plastid transformation vectors have been matured and thus novel vector designs offer accomplishment of new molecular arrangements that make possible versatile transformation and expression approaches. Among these developments, two plastid transformation vectors are highlighted. First, the "operon-extension" vectors, which rely on the extension of endogenous operons, which implies that the vector does not contain promoter and regulatory elements required for translation (Herz et al. 2005). By eliminating the need for a promoter in the insertional DNA, the risk of leading to undesirable rearrangements in the plastome, mediated by recombination with endogenous sequences, is avoided (Herz et al. 2005). Targeting transgene insertion at a highly transcribed operon is desirable to favor high yields. Among the well-characterized operons meeting this criterion are those coding for the adenosine triphosphate (ATP) synthase (*atpB/atpE*) or the D1 polypeptide of the photosystem II (*psbA*; Deng and Gruissem 1987). Under this modality, endogenous genes and untranslated regions (UTR) from a particular operon may serve as flanking regions at the vector that act as recombination sites (Fig. 4.2).

Another configuration consists of using at least two deconstructed vectors, which contain modules that constitute precursors of the desired expression cassette. Co-transformation of plastids with these separated modules leads to the insertion of the individual modules, with a subsequent rearrangement mediated by homologous recombination that leads to assembly of the desired expression cassette (Herz et al. 2005). These "split" transformation vectors allow for achieving different expression cassettes with a wide variety of genes of interest under a diversity of control elements. This strategy can be followed to yield promoter-containing or operon-extension configurations (Fig. 4.3).

In terms of biosafety, a growing concern from both regulatory agencies and the public in certain countries comprise the notion of a significant risk of having non-target effects of the antibiotic resistance enzymes expressed in the GMO, which may interfere with antibiotic therapies. Other contemplated risks consist of the possible horizontal transfer of marker genes to bacterial species at the human gut, causing new antibiotic-resistant strains of clinical relevance (Miki and McHugh 2004). Considering that several antibiotic-resistance marker genes have been used thus far (Table 4.1), and that about 10% of the recombinant proteins produced corresponds

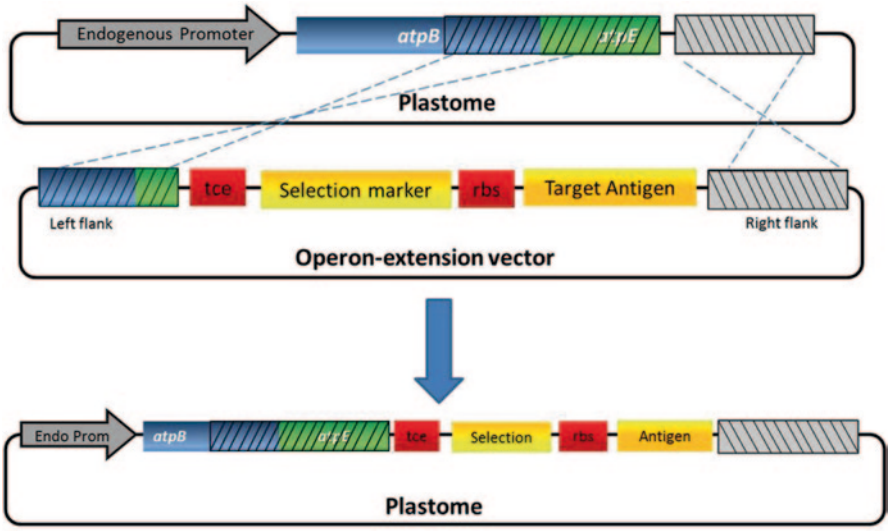


Fig. 4.2 General representation of integration of foreign genes into chloroplast genome by an operon-extension vector. Graphic representation of an operon-extension vector comprising of transcription control elements (*tce*), ribosome binding site (*rbs*) and the genes coding for the antigen and the selection marker, between *atpB* and *atpE* as *left* and *right* flanks, respectively, whose endogenous promoter drives transgene expression. *Light blue crossed lines* symbolize homologous recombination events

to the marker protein (Maliga 2002), further studies to determine the safety of each marker protein are needed. Another concern is the gene flow between plants and the acquisition of resistance to wild-type endemic crops. Therefore, molecular strategies allowing for the elimination of the selection marker are of importance for the molecular farming field and distinct approaches to achieve this objective are available: (1) A CRE-*loxP* system has been applied to excise markers on stable transformants (Corneille et al. 2001), which is based on the addition of recombinase-recognition sites (*loxP*) flanking the selectable marker. The expression of a *Cre* gene is subsequently induced by a transient expression approach (Lutz et al. 2006), inducing a *loxP*-mediated excision of the marker gene in a site-specific recombination event. (2) The use of short direct repeats flanking the marker gene has been successfully applied by Iamtham and Day (2000). (3) The use of co-integrated vectors has been proposed by Klaus et al. (2004), where one vector having a single homologous recombination and a marker gene located outside of the recombination region allows for the selection of transformants, but in a subsequent step generates an unstable arrangement due to the presence of direct repeats, leading to gene marker excision. Therefore, molecular tools for developing transplastomic plants devoid of selection markers constitute an attractive alternative for the field of plant-based vaccine development (Corneille et al. 2001).

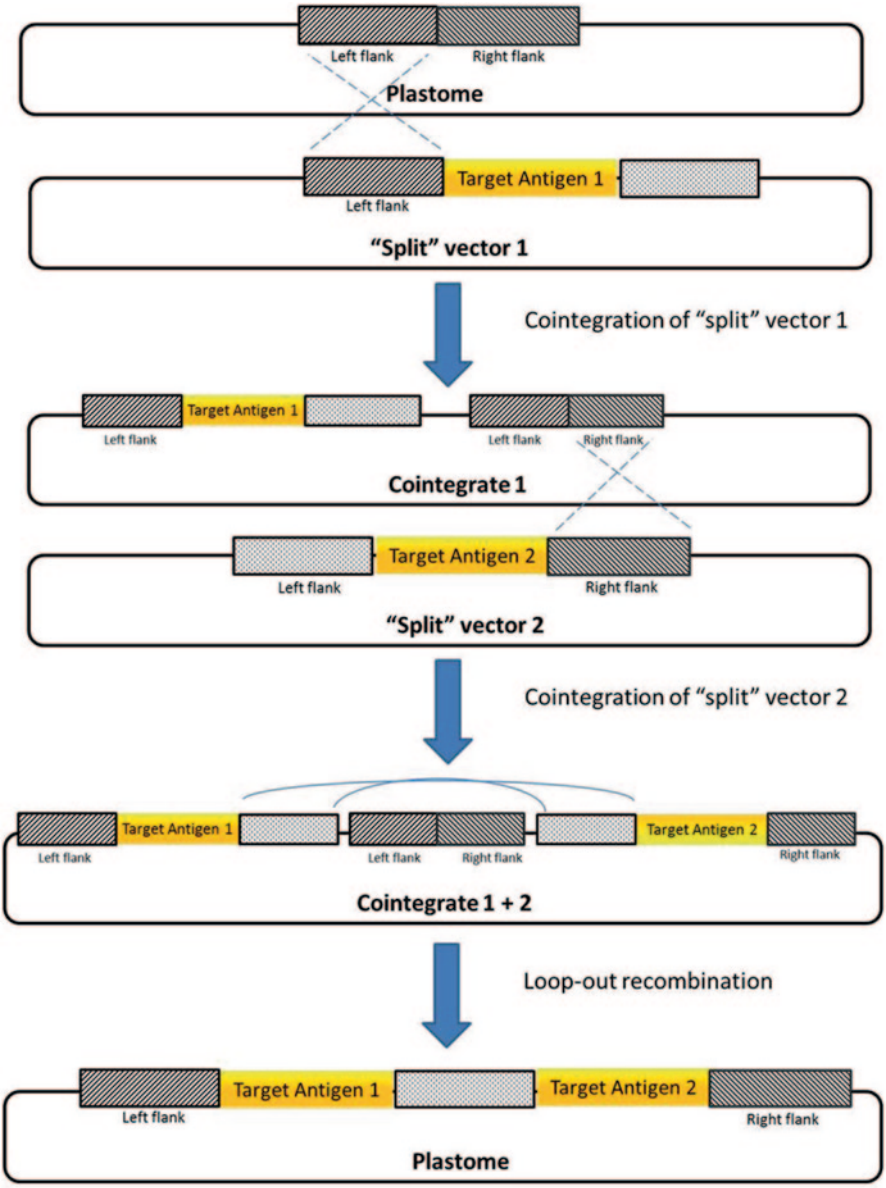


Fig. 4.3 General representation of integration of foreign genes into chloroplast genome by a "Split" transformation vector. Separated modules of two "split" vectors with only one flanking region (*diagonal striped boxes*), a target gene coding for the antigen or selection marker, and a common region between both vectors (*point box*). *Light blue crossed lines* symbolize homologous recombination events

Table 4.1 Reporter and selectable marker genes expressed via the chloroplast genome

Reporter/selectable genes	Organism	Ref.
cat—chloramphenicol acetyl transferase	(a) Cucumber etiopasts (T)	(a) Daniell and Mc Fadden 1987
	(b) Cultured tobacco cells (T)	(b) Daniel et al. 1990
uidA—β-glucuronidase	Wheat leaves and calli (T)	Daniell et al. 1991
aadA—aminoglycoside adenylyl transferase	(a) Chlamydomonas (S)	(a) Goldschmidt et al. 1991
	(b) Tobacco (S)	(b) Svab and Maliga et al. 1993
nptII—neomycin phosphotransferase	Tobacco (S)	Carrer et al. 1993
aphA-6—aminoglycoside phosphotransferase	(a) Chlamydomonas (S)	(a) Batema and Purton et al. 2000
	(b) Tobacco (S)	(b) Huang et al. 2002
gfp—green fluorescent protein	(a) Tobacco and Arabidopsis (T)	(a) Hibberd et al. 1998
	(b) Potato (S)	(b) Sidorov et al. 1999
aadA-gfp-fusión protein	Tobacco (S) and rice (T)	Khan and Maliga 1999
Badh—betaine aldehyde dehydrogenase	Tobacco (S)	Daniell et al. 2001

T transient expression, *S* stable integration

Gene Transfer Methods

Polyethylene Glycol (PEG)-Mediated Method

The chloroplast genome of higher plants can be modified by two different techniques: the biolistic method and the PEG-mediated method. For the PEG-mediated method, isolated protoplasts (plant cells without cell wall) are treated with PEG in the presence of the transformation vector. In a set of previous experiments, our group has demonstrated that both methods are equally efficient in *Nicotiana tabacum*. In contrast to the particle gun, the PEG-mediated transformation of plant cells does not require expensive equipment such as particle guns.

Besides *N. tabacum* (Golds et al. 1993), PEG-mediated plastid transformation has been achieved in several different plants, including *N. plumbaginifolia* (O'Neill et al. 1993), *Solanum lycopersicum* (tomato; Nugent et al. 2005), *Brassica oleracea* var. botrytis (cauliflower; Nugent et al. 2006), and *Lactuca sativa* (lettuce; Lelivelt et al. 2005). Reliable protoplast regeneration is necessary in all cases.

PEG-mediated transformation in plants also requires the enzymatic removal of the cell wall which is a barrier for the DNA entry. Once protoplasts are isolated, they can be mixed with PEG in the presence of DNA. Although more than 15 years have passed, the exact mechanism of how DNA is transported through the double membrane of chloroplasts is still unknown (Spörlein et al. 1991; Koop et al. 2007).

Our group has published several detailed protocols and one review focused on PEG-mediated plastid transformation in tobacco (Koop et al. 1996; Dovzhenko

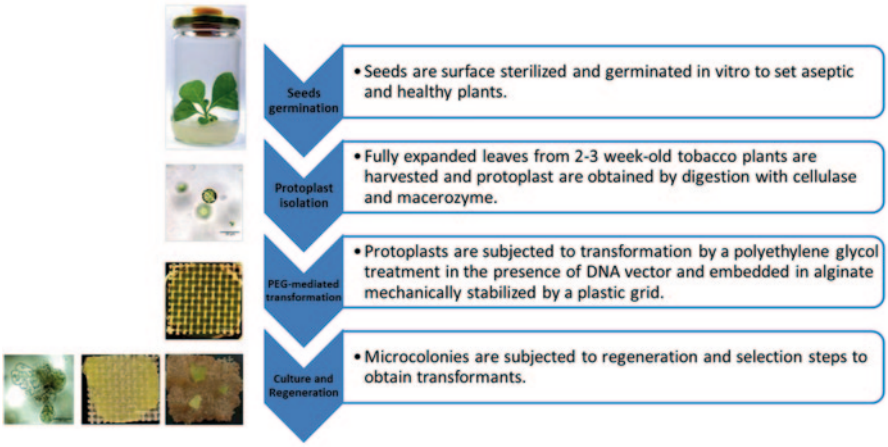


Fig. 4.4 Workflow of PEG-mediated transformation method

et al. 1998; Kofer et al. 1998). High transformation efficiency (up to 20–50 plastid transformants per 10^6 protoplasts) has been achieved. Figure 4.4 presents a general work flow for this method.

Biolistic Method

The biolistic transformation method consists of coupling the expression vector DNA with microprojectiles of tungsten or gold, which are subsequently shot at a high pressure onto the plant tissues. One critical step is the coating process, comprising an incubation of DNA/microprojectile mixture with calcium chloride and spermidine, a cationic polyamine (Sanford et al. 1993) that protects DNA from degradation by the cellular nucleases, shields the negative charges of the DNA phosphate backbone, and allows for hydrophobic interactions with the particles (Brune et al. 1991; Thomas et al. 1996). After this incubation, particles are washed several times with ethanol. The DNA-particle suspension is pipetted onto a "macrocarrier" that allows for assembling the gene gun system. The helium pressure and the distance between the macrocarrier and the target tissues are considered the primary conditions to be optimized for a specific target. Figure 4.5 presents a general work flow for this method. Manipulation of the chloroplast genome has become a routine in the model system tobacco and has been extended to edible solanaceous crops, including potato and tomato (Ruf et al. 2001; Zhou et al. 2008; Apel and Bock 2008; Valkov et al. 2011; Scotti et al. 2011).

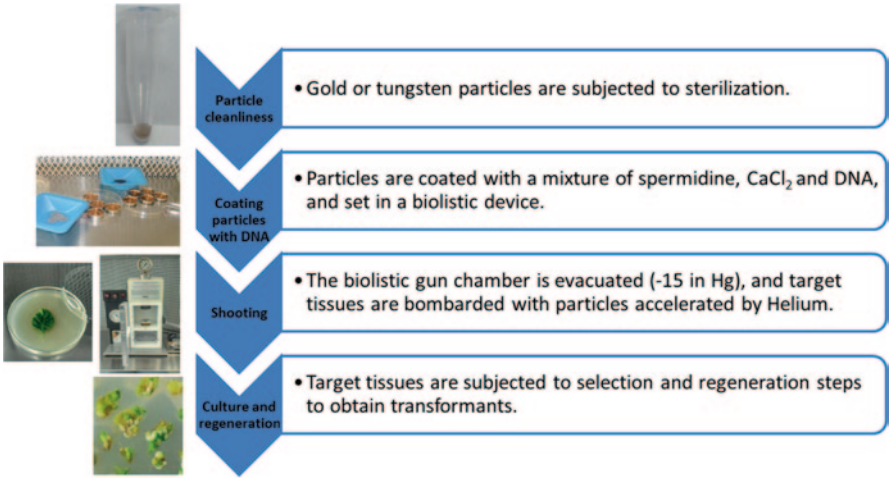


Fig. 4.5 Workflow of biolistic-mediated transformation method

Achievements on the Chloroplast-Derived Vaccines Field

Chloroplast-based expression has become a well-explored approach in the field of plant-based vaccines. Several biopharmaceutical proteins have been produced by means of transplastomic technologies (Table 4.2). The following subheadings consist of the description of representative examples that illustrate the potential of this technology to accomplish advancements in vaccination models against relevant infectious diseases (see Box 4.1).

Anthrax

Anthrax vaccine for human use is currently derived from supernatants of *Bacillus anthracis* cultures containing the protective antigen (PA) and traces of the lethal and edema factors that contribute to adverse side effects. Therefore, an effective expression system providing a clean, safe, and effective vaccine is needed. In an effort to produce an advantageous anthrax vaccine, in 2005, Koya et al. expressed PA in transgenic tobacco chloroplasts by inserting the *pagA* gene into the chloroplast genome. Yields in mature leaves reached PA levels of up to 14.2% TSP. Cytotoxicity measurements in macrophage lysis assays showed that chloroplast-derived PA was equal in potency to PA produced in *B. anthracis,* suggesting that this platform is highly efficient for the production of the functional PA antigen. Researchers explored the immunogenic potential of this candidate vaccine by immunizing mice subcutaneously with partially purified chloroplast-derived PA or *B. anthracis*-derived PA along with an adjuvant. This procedure elicited immunoglobulin G (IgG) titers up to 1:320,000

Table 4.2 Biopharmaceutical proteins expressed via the chloroplast genome

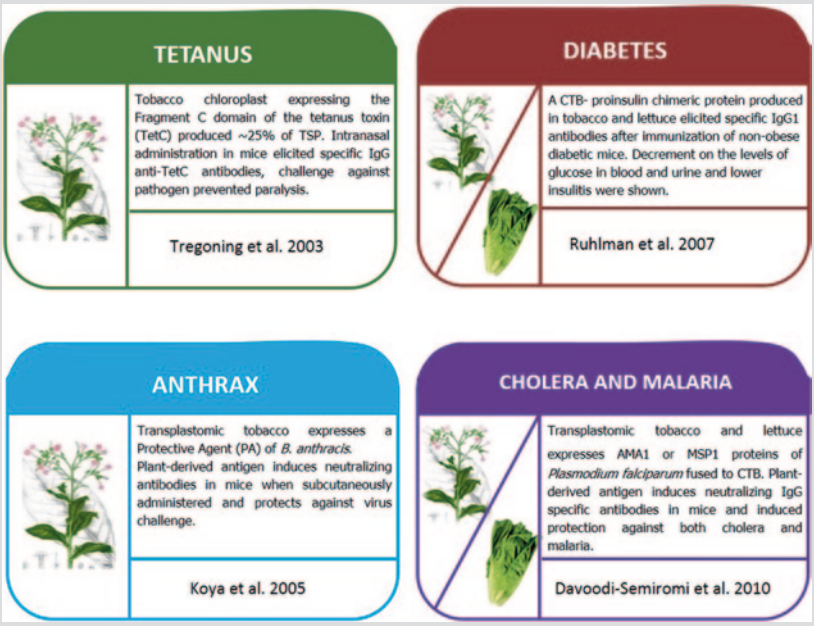
Biopharmaceutical proteins	Gene	Site of integration	Promoter	5′/3′ regulatory elements	Ref.
Elastin derived polymer	EG121	*trnI/trnA*	*Prrn*	*T7gene10/ TpsbA*	Guda et al. 2000
Human somatotropin	hST	*trnV/rps12/7*	*Prnn, PpsbA*	*T7gene10 or psbA/Trps16*	Staub et al. 2000
Cholera toxin	CtxB	*trnI/trnA*	*Prrn*	*ggagg/TpsbA*	Daniell et al. 2001
Antimicrobial peptide	MSI-99	*trnI/trnA*	*Prrn*	*ggagg/TpsbA*	DeGray et al. 2001
Insulin like growth factor	IGF-1	*trnI/trnA*	*Prrn*	*PpsbA/TpsbA*	Daniell et al. 2009
Interferon-α-5	INFα5	*trnI/trnA*	*Prrn*	*PpsbA/TpsbA*	Torres 2000
Interferon-α-2B	INFα2B	*trnI/trnA*	*Prrn*	*PpsbA/TpsbA*	Arlen et al. 2009
Human serum albumin	Hsa	*trnI/trnA*	*Prnn, PpsbA*	*ggagg, psbA/ TpsbA*	Fernández-San Millan et al. 2003
Interferon-γ	IFN-γ	*rbcL/accD*	*PpsbA*	*PpsbA/TpsbA*	Leelavathi et al. 2003
Monoclonal antibodies	Guy´s13	*trnI/trnA*	*Prrn*	*ggagg/TpsbA*	Daniell et al. 2001
Anthax protective antigen	Pag	*trnI/trnA*	*Prrn*	*PpsbA/TpsbA*	Watson et al. 2004
Plague vaccine	CaF1-LcrV	*trnI/trnA*	*Prrn*	*PpsbA/TpsbA*	Singleton 2003
Canine parvovirus vaccine	CPV VP2	*trnI/trnA*	*Prrn*	*PpsbA/TpsbA*	Molina et al. 2004

and both groups of mice showed 100% survival after a challenge with lethal doses of anthrax toxin. Authors estimate that the yields of 150 mg of PA per plant allow for the production of 360 million doses of a purified vaccine free of bacterial toxin contamination by cultivating 1 acre of land. Therefore, this chloroplast-based model is a promising approach and it is expected to result in a positive impact in the field of vaccination against this bioterrorism agent.

Tetanus

In 2003, Tregoning et al. reported the production of a mucosal tetanus vaccine in tobacco consisting of the fragment C of the tetanus toxin (TetC), which is a non-toxic 47-kDa polypeptide fragment able to elicit protective immune responses via parenteral immunization. Conventional platforms for its production consist of *Escherichia coli,* yeast, and insect cells. The expression in chloroplasts was compared when the native or a synthetic gene was used, reaching expression levels of 25 and 10% TSP, respectively. Test mice immunized intranasally with the chloroplast-derived TetC developed specific IgG antibodies and were protected against paralysis. This comprises an important approach to induce protection against this pathogen; thus, it is considered a potential candidate for clinical trials.

Box 4.1 Representative advanced plant-based candidate vaccines developed by means of transplastomic technologies



Diabetes

The B subunit of the toxin from *Vibrio cholera* (CTB) is recognized as one of the most potent mucosal adjuvants; in this context, various antigens have been targeted by means of using CTB as carrier under a genetic fusion, leading to the production of chimeric proteins. Considering the urgent need for developing immunotherapies against type I diabetes, Ruhlman et al. (2007) designed a fusion protein fusing CTB with human proinsulin (CTB-Pins), which was expressed in lettuce and tobacco transplastomic lines.

This research group showed that the tobacco and lettuce transplastomic lines accumulated CTB-Pins at levels of up to ~16 and ~2.5% TSP, respectively. Immunization of non-obese diabetic (NOD) mice allowed for the characterization of the therapeutic effect. The pancreas of CTB-Pins-immunized mice showed decreased infiltration of lymphocytes (insulitis). Moreover, insulin-producing β-cells in the pancreatic islets of CTB-Pins-immunized mice showed no important changes whereas few β-cells were detected in the pancreatic islets of the negative controls. Lower blood or urine glucose levels were also detected in CTB-Pins-immunized mice. IgG1 levels were predominant in the experimental group, suggesting that T-helper 2 (Th2)-lymphocyte-mediated oral tolerance is the mechanism behind the prevention of pancreatic insulitis and the preservation of insulin-producing β-cells.

The relevance of this development is given in part by the use of an edible crop because the typical platform consisting of tobacco chloroplast transformation limits the oral delivery of vaccines due to the presence of toxic compounds, such as alkaloids (Baldwin 1988). This development opens up the possibility for the low-cost production and oral delivery of vaccines with no purification process based on the use of a transplastomic edible crop.

Cholera and Malaria

Cholera and malaria are major diseases of high mortality, in particular in low-income countries. The available cholera vaccine is expensive, immunity is lost in children less than 3 years old, and adults are not fully protected. On the other hand, there is no vaccine yet available for malaria.

Based on the use of chimeric proteins comprising the CTB as carrier, Davoodi-Semiromi et al. reported in 2010 the chloroplast-based production of malaria apical membrane antigen-1 (AMA1) and the surface of merozoite protein 1 (MSP1) in chloroplasts. Besides the conventional model of tobacco, lettuce transplastomic lines were also developed in order to assess the expression of the chimeric proteins. The researchers showed that fusion proteins CTB-AMA1 and CTB-MSP1 accumulated in tobacco at levels of 13.17 % TSP, whereas in the case of lettuce, accumulation reached 7.3 and 6.1 % TSP, respectively. In order to evaluate the immunogenicity of these chlororoplast-made antigens, groups of mice ($n = 10$) were immunized subcutaneously or orally with purified antigens from transplastomic tobacco leaves.

Significant levels of specific IgG1 antibody titers anti-MSP1 were elicited in immunized mice, showing cross-reactivity with native parasite proteins on Western blots and immunofluorescence studies. Challenge studies against *Plasmodium* showed 100 % of protection against the parasite entry to erytrocytes. Cholera toxin challenge revealed that both orally and subcutaneously immunized mice showed protection levels of 100 and 89 %, respectively, which correlated with specific levels of serum and intestinal anti-CTB IgA in the orally immunized group. Therefore, dual immunity against two major diseases, cholera and malaria, is provided by this chloroplast vaccine, representing another candidate that may be evaluated in clinical trials.

Prospective View

Under the light of the state of the art represented in this chapter, distinct perspectives raised for the plastid-based expression strategies applied to vaccine development. It is clear that these technologies represent a relevant choice for the field of producing plant-derived vaccines. In terms of advantages, the most prominent consists of the high levels of the recombinant protein produced in comparison to other expression strategies, which are in general terms ten to hundred times higher than those reached by conventional nuclear-based expression approaches. The biosafety

offered by the system is singular as maternal inheritance of the transgene takes place for most of the plant species. Due to the presence of the efficient homologous recombination machinery in chloroplasts, transgene insertion is site directed and the availability of chloroplast genome sequences for a number of species makes this a viable and versatile tool.

Foreign proteins synthesized in chloroplasts have access to some post-transcriptional modifications, including disulfide bonds, with the notable exception of glycosylation (Daniell et al. 2005). This in fact may constitute an advantage to express proteins in which plant glycosylation pattern leads to undesired characteristics such as allergenicity. However, proper glycosylation is in fact a requirement for the stability and functionality of many human therapeutic proteins, and thus are not considered appropriate targets for this technology, at least under the current outlook. In this regard, strategies for addressing this limitation are envisioned. Although not a chloroplast-based expression approach, it has been suggested the implementation of approaches based on the export of nuclear-encoded proteins into the chloroplast; thus, they can serve as storage compartments of proteins that have been previously processed by the maturation machinery of endoplasmic reticulum / Golgi which includes glycosylation. Although this pathway allowing protein traffic from the endomembrane system to chloroplasts has been evidenced, further reports confirming and characterizing in detail this mechanism may lead to a new perspective for this field (Villarejo et al. 2005; Faye and Daniell 2006; Gomord et al. 2010).

On the other hand, it should be noted that plant chloroplast transformation is a laborious task. For tobacco, it takes about 5 months to obtain primary transformants. This is mainly due to the various selection/regeneration cycles needed for reaching the homoplastomic state, which means that homogeneity in the transgenic state has been attained in the multicopy plastome. In the field of plant-chloroplast genetic engineering, tobacco is the plant species typically taken as the initial model because it is essentially the only plant in which chloroplast transformation protocol is highly reproducible and efficient (one to five stable transformants per bombarded leaf). However, this system requires a complex downstream processing as purification is definitively needed to yield a product free of the inherent toxic compounds present in the host, such as alkaloids (Baldwin 1988).

To date, several crop chloroplast genomes have been transformed (cauliflower, cabbage, lettuce, potato, tobacco, tomato, carrot, rice, and soybean among others); thus, an expansion of the vaccine candidates tested by means of transplastomic technologies is expected in the following years. During the past decade, lettuce transformation has been established as a robust platform for plastid transformation and vaccine development, representing an advancement of particular relevance as it constitutes an edible plant. This procedure takes about 3 months for generating primary transformants under a lower efficiency than that of tobacco: one to three stable transformants per ten bombarded leafs. At present, the following antigens have been produced in lettuce: dengue-3-premembrane and envelope polyprotein, CTB-malaria antigen, and CTB-proinsulin antigen (Kanagaraj et al. 2011; Davoodi-Semiromi et al. 2010; Ruhlman et al. 2007). The development of these initial models based on an edible crop has opened the door for the production of oral vaccines

without the need for extensive processing, in contrast with those obtained in tobacco having intrinsic toxic compounds. This reduced number of examples map out a field of opportunity for the development of new candidates with the attributes offered by transplastomic approaches and a source of edible biomass. The availability of efficient protocols for other edible crops will be a positive aspect that will play a key role in the field. In this regard, one interesting expression host is carrot, which has been successfully targeted by transplastomic technologies (Kumar et al. 2004) and could be used as cell suspension cultures propagated in bioreactors, as in the case of the glucocerebrosidase produced by Protalix (Carmiel, Israel) by a nuclear-based expression approach. It has been approved under the name of ELELYSO or UPLYSO as an enzyme indicated for long-term enzyme-replacement therapy for adults with a confirmed diagnosis of type 1 Gaucher disease. These products have received, during 2012 and 2013, marketing authorization from the Israeli Ministry of Health, Ministry of Public Health in Uruguay, and the Brazilian National Health Surveillance Agency (ANVISA), while the US Food and Drug Administration granted approval for ELELYSO as long-term enzyme-replacement agent (Protalix 2013).

Of special attention is the fact that, despite the several advantages attributed to transplastomic approaches, the current industrial adoption has advanced in a higher degree for those systems based on nuclear transient expression, such as agroinfiltration with proviral vectors and conventional transgenic cell lines (Yusibov et al. 2011). It is also expected that the reported developments associated with diverse pathogens would serve as a precedent for the exploration of edible crops, comprising vaccine candidates against malaria, cholera, tetanus, anthrax, plague, and amoebiasis. An interesting unexplored focus consists of taking advantage of expressing multiple genes by operon-like arrangements, where a single promoter and transformation event may allow for the transcription of polycistrons able to yield distinct polypeptides (Arai et al. 2004; Nakashita et al. 2001). This strategy is proposed as a convenient path for the development of multivalent or multicomponent vaccines, and thus vaccines of broad immunoprotective potential may be developed. Simultaneous expression of polypeptides with adjuvant activity may also offer a mechanism for enhancing immunogenicity. It is expected that applying these approaches will aid in the development of innovative plant-based vaccines.

Therefore, the future of vaccines based on transplastomic technologies augurs low-cost alternative approaches for immunization to combat infectious or even noncommunicable diseases, avoiding inactivated pathogens, purification, cold chain, and injections, thus representing low-cost and safe vaccines.

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Chapter 5
Seed-Based Expression Strategies

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Introduction

Since the first report of a transgenic plant expressing a recombinant viral antigen was presented back in the 1990s (Mason et al. 1992), the concept of developing plant-based vaccines has become widely popular as an attractive system for biopharmaceutical recombinant protein production. In particular, low-cost subunit vaccines are widely needed for global immunization against diseases that can be readily preventable by vaccination (Tiwari et al. 2009). Although there are several platforms for the production of these subunit vaccines, including the use of Chinese hamster ovary (CHO) cells and yeasts in bioreactors, the use of plants as a production platform is highly desirable. Plants are capable for processing complex proteins properly, allowing for the retention of native biological activity in the recombinant protein. In addition, plants offer low-cost, safe, and environmentally friendly platform for subunit production as they do not transmit mammalian pathogens when properly cultivated (Oszvald et al. 2008; Wu et al. 2007). To date, several recombinant immunogens have been successfully produced in different plant systems and in different plant tissues, including leaves, roots, tubers, and seeds. Efforts in subunit vaccine production in plants have received US Department of Agriculture (USDA) approval and are undergoing clinical studies, such as the Newcastle disease vaccine (for poultry) expressed in tobacco cell suspension cultures (Boothe et al. 1997; Mihaliak et al. 2005; Yusibov et al. 2011). In addition, plant-based subunit vaccines can be delivered orally, and without the use of needles, thereby reducing costs

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required for administration of these vaccines, as well as costs associated with the required number of personnel or health care professionals for proper delivery and patient compliance. Moreover, subunit vaccines produced in some plant systems (such as soybean and rice, among others) can be stored at room temperature for long durations without loss of activity, thus alleviating the need for refrigeration. All these collective features will contribute towards mitigation of large capital investments by developing countries that cannot afford these costs.

The first "proof of concept" of the production of a plant-based vaccine was reported by Haq et al. (1995) wherein the B subunit of the *Escherichia coli* heat labile enterotoxin (LTB) was synthesized in both tobacco and potato. However, these early efforts yielded low levels of expression of the LTB antigen following freeze-drying to aid in determining the administration of reliable dosages of the antigen (Rosales-Mendoza et al. 2008). Since then, different approaches for vaccine design and production have been proposed and evaluated. Over the years, more advanced strategies for antigen production in plants have been developed, including targeting protein accumulation to different cellular compartments such as chloroplasts, endoplastic reticulum (ER), and cytosol. In addition, tissue-specific expression of foreign genes has also provided means for improving yields of recombinant proteins produced in plant systems (Zhang et al. 2008). In some cases, targeting to cellular organelles has resulted in higher levels of protein accumulation compared to cytosolic targeting. For example, proteins can be retained in the ER by the inclusion of retention signal on the antigen design. This approach overcomes unstable posttranslational modifications in plant cells due to trafficking and storage destination of the protein (Garg et al. 2007; Piller et al. 2005). In this context, seeds have emerged as desirable plant organs for expression of recombinant proteins in plants.

Characteristics of Seeds and their Advantages as Platforms for Molecular Pharming

Among different plant tissues, seeds serve as important systems for mass propagation and for delivery of food nutrients, as well as for synthesis of value-added products for both agricultural and industrial purposes. Seed propagation, packing, storage, and distribution technologies are well developed and established for most seed crops.

As value-added products, seeds serve as promising hosts for producing pharmaceuticals and therapeutic proteins and offer significant advantages over transgenic animals and mammalian cell cultures. These include the following:

Yield Seeds are natural reservoirs of proteins, carbohydrates, lipids, as well as of other important metabolites required for seedling growth and development. As seeds contain good amounts of protein, they serve as well-suited organs for protein

synthesis and storage. Thus, they are deemed useful as storage reservoirs of valuable recombinant proteins (Boothe et al. 1997; Karg and Kallio 2009). Moreover, seed production can be easily and relatively cheaply increased under field conditions. As an example of productivity, recombinant protein production in maize seed can be rapidly scaled up. With a generation time of 3–4 months, depending on the cultivar, along with a yield of several thousands of seeds per generation, recombinant protein production can be scaled up to several hundreds of grams (anywhere between 200 g and 2 kg) per acre (Hood et al. 1999; Tacket et al. 2004). The potential of using maize for large-scale production of therapeutic recombinant proteins that maintain integrity and biological activity has been well documented (Hood et al. 1999; Tacket et al. 2004). Both corn and rice have the highest annual grain yield (FAO 2013) with well-established genetic transformation protocols available for introducing foreign genes coding for valuable antigenic proteins (Stoger et al. 2005).

Stability Seed-produced heterologous proteins are highly stable due to low moisture content of mature seeds, proper protein folding, and low cytoplasmic proteolytic degradation, resulting from the presence of a rich group of molecular chaperones and disulfide isomerases in developing seed (Stoger et al. 2005). Recombinant proteins can be stored in protein bodies of the endosperm, escaping proteolysis during maturation in the cytosol, and subsequent programmed cell death during the final stages of cereal grain maturation (Wu et al. 2007). This allows for storage of large quantities of seeds, containing recombinant proteins, at room temperature for over several years (Moravec et al. 2007; Wu et al. 2007). Moreover, this also contributes to increased biomass production of stable antigenic proteins without undergoing processing for purification and extraction (Moravec et al. 2007; Wu et al. 2007). This is in contrast to antigenic proteins that are expressed in leaf tissues that tend to be unstable, with a limited shelf life, and thus would require immediate processing following harvest (Fischer et al. 2004).

Processing Versatility Cereals are attractive production systems of recombinant proteins as they are low-cost crops, and are consumed by both animals and humans (Piller et al. 2005). Common commercial seed crops include maize (or corn), rice, soybean, wheat, peanut, and pea. For example, corn and rice are rich sources of carbohydrates and proteins; moreover, these cereals are staple crops grown in many parts of the world and serve as primary sources of dietary food intake in many cultures and in different countries. For these reasons, they have been used as platforms for the production of subunit oral vaccines (Oszvald et al. 2008). Cereals can be processed into feed, meal, flour, milk, or other consumables using different methods and can serve as good candidate sources of immunogenic proteins, thus facilitating formulation, dosage, and delivery of subunit oral vaccines (Garg et al. 2007; Moravec et al. 2007). Protein purification is also facilitated in seed-based systems due to low contents of phenolic compounds and pigments, and with lower complex mixtures of proteins and lipids when compared to those of leaves (Daniell et al. 2001; Tiwari et al. 2009).

Vaccine Delivery Many seeds are edible and may be orally administered with no toxic effects, allowing for opportunities in developing ready-made vaccine formulations based on seed biomass and with minimal processing (Moravec et al. 2007; Ma et al. 2003; Stoger et al 2005; Daniell et al. 2001; Takagi et al. 2005; Muntz 1998; Vimolmangkang et al. 2012). This approach will dramatically lower production costs compared to parenteral vaccine production. In addition, induction of mucosal and systemic immune responses can be elicited when oral route delivery is opted for antigen administration. This is of particular significance as this component of the immune system can provide a first line of defense to combat infection at the site of invasion of various pathogenic organisms. As substantial degradation is a potential risk for orally administered vaccines due to the nature of the gastrointestinal tract, delivering the immunogen within the context of plant biomass can delay proteolysis, thus improving bioavailability of the subunit vaccine to the mucosal immune system. This effect is termed as bioencapsulation (Streatfield 2006).

The above-mentioned characteristics of seeds clearly support their use as low-cost and convenient platforms for the production of subunit vaccines. To date, subunit vaccine antigens (Hayden et al. 2012; Hudson et al. 2013; Nochi et al. 2007; Streatfield and Howard 2003; Yang et al. 2011; Yuki et al. 2012; Vimolmangkang et al. 2012) and therapeutic antibodies (Stoger et al. 2000) have been successfully expressed in plant seeds. Among seed-based vaccine candidates reported thus far, a number of them have been evaluated in test animals and even in humans to assess immunogenicity and/or immunoprotection upon oral, intraperitoneal (i.p.), or intramuscular administration (Chikwamba et al. 2002; Karaman et al. 2012; Lamphear et al. 2004; Moravec et al. 2007; Nojima et al. 2011; Qian et al. 2008; Tacket et al. 2004; Vimolmangkang et al. 2012; Wang et al. 2009; Wu et al. 2007; Zhang et al. 2008).

Distinct Modalities Allow for Targeting Vaccine Production in Seeds

Oil Bodies-Based Approaches

Oil bodies are subcellular organelles present in many plant species and serve as storage sites for triacylglycerides (TAGs; Fig. 5.1). These compounds are particularly abundant in oilseeds whereby TAGs represent the primary energy reserves supporting germination and early seedling growth (Boothe et al. 1997). Interestingly, oil bodies from oilseeds, such as soybean, saffron, maize, cotton, rice, rapeseed, and sunflower can be used as target sites for accumulation of recombinant proteins. Model species, such as *Arabidopsis thaliana,* have been used in initial efforts to demonstrate production of oil bodies-directed proteins. In this context, relevant model proteins are oleosins, which are hydrophobic plant proteins associated with

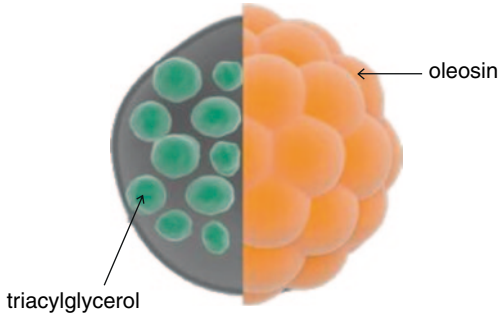

Fig. 5.1 Schematic representation of an oil body. Oil bodies are organelles that serve as the storage for *triacylglycerides* (*TAGs*) as the primary energy reserve of the seed. TAGs are surrounded by proteins known as *oleosin*

oil bodies (Capuano et al. 2007). It is reported that oleosins cover entire surfaces of oil bodies, and account for 2–8% of total seed proteins in oilseeds (Huang 1992). Proper refolding of proteins spontaneously occurs when these are expressed in oil bodies (Parmenter et al. 1995). Therefore, oleosins can be used to design strategies that will direct unrelated proteins to these subcellular targets. For instance, a desirable protein of interest can be expressed as an oleosin fusion protein wherein it can be covalently targeted to oil bodies. In addition, presence of a fusion protein along surfaces of oil bodies aid in protein extraction, as oil bodies can be separated from other cellular components by either floatation or centrifugation, thus eliminating the need of costly and time-consuming chromatographic steps involved in purification (Bhatla et al. 2010; Capuano et al. 2007; Parmenter et al. 1995).

Subunit vaccines comprising recombinant oil bodies can be used to elicit an immune response against an antigen via any route of administration, including transdermal and mucosal (further detail is presented in Chap. 2). This type of formulation confers enhanced immunogenicity when compared with conventional formulations as plant lipids can also serve as adjuvants (Capuano et al. 2007; Deckers et al. 2004).

Most of these seed-based platforms have been explored by Sembiosys (www.sembiosys.com). In a pioneering report, a transferrin-binding protein B (TbpB) *N*-lobe gene from *Neisseria meningitidis* M982 strain was expressed and targeted onto surfaces of oilseed *Arabidopsis*. Oil bodies displayed the antigenic fusion protein along the surface. Groups of test mice were immunized with antigen complexes, and specific antibody responses have been elicited, thus serving as a promising candidate vaccine (Deckers et al. 2004 Patent US6761914 B2).

A recombinant human precursor insulin (Des-B) fused with oleosin has also been expressed in *Arabidopsis,* and plant-derived insulin accumulated to significant levels in transgenic seeds (0.13% of total seed protein). Expression of insulin in *Arabidopsis* seeds was sixfold higher than that previously reported in plants. The biological activity of recombinant insulin was demonstrated *in vivo* using an insulin tolerance test in mice. Results showed that at doses of 1 U/kg body weight, the recombinant protein was equally effective in lowering blood glucose as that of the

commercially available Roche insulin. Moreover, the plant-derived insulin did not elicit any observed toxic side effects (Nykiforuk et al. 2006).

Protein Bodies and Protein Storage Vacuoles

The storage protein content in seeds is approximately 25% of the dry weight (Schmidt 2013). These storage proteins are accumulated as aggregates, either in the form of protein bodies (PBs) or protein storage vacuoles (PSVs), in cells (Fig. 5.2). PBs are derived from the ER, in particular, these are formed in the rough ER and then migrate to the cytosol. When reaching adequate size, protein bodies are sequestered by PSVs through autophagy (Khan et al. 2012; Fig. 5.2). Interestingly, PBs and PSVs have been used as protein accumulation targets along with the use of strong seed-specific promoters, and also by fusing endogenous signal peptides to N terminals of antigens of interest, thus successfully targeting these subcellular structures (Fig. 5.3). In addition to increased yields, these immunogens are also protected against proteolysis, thus contributing to additive effects to those exerted by cell walls, thus increasing the gut-associated lymphoid tissue (GALT)-delivery rate (Nochi et al. 2007). Developing a better understanding of sorting and accumulation of proteins is critical for engineering production of heterologous proteins in seeds.

Lau et al. (2010) produced Merozoite surface protein 1 (MSP 1) of *Plasmodium falciparum,* an important virulent pathogen that causes human malaria, in *Arabidopsis* seeds. Using the strong seed-specific phaseolin promoter to drive the transcription of MSP1, Arabidopsis seeds accumulated the recombinant protein at levels of 5% of total extractable protein. Immune sera from human patients that had been infected with *P. falciparum* were used to evaluate the antigenicity of *Arabidopsis*-derived MSP1 and a positive immunodetection was observed. This demonstrated that antigenic determinants have been conserved in the plant-derived protein. The recombinant protein was targeted to PSVs by either adding the vacuolar targeting signal of phaseolin or fusing it along with a stable plant storage protein. These strategies resulted in dramatic increase in levels of protein expression. Thus, these studies could serve well in the development of a low-cost malaria vaccine, although immunogenic properties of these candidate vaccines are yet to be demonstrated.

Recently, Yang et al. (2012) reported on the expression of an allergen *Dermatophagoides farinae* group 2 (Der f 2) from house dust mite (HDM), driven by a glutelin endosperm-specific promoter fused to a glutelin B-1 (GluB-1) signal peptide, in transgenic rice. Protein derivatives aggregated in the ER, and these were deposited in protein body-like structures. Mice orally immunized with this transgenic rice indicated that Der f 2 bodies suppressed production of Der f 2-specific IgE and IgG. Moreover, an allergen *Betula verrucosa* (Bet v 1) from birch pollen, fused to a GluB-1 signal peptide and a KDEL ER retention signal, was also expressed in rice, and the recombinant protein was found to be deposited in large PBs, at accumulation levels of 207 µg per grain (Fig. 5.4; Wang et al. 2013).

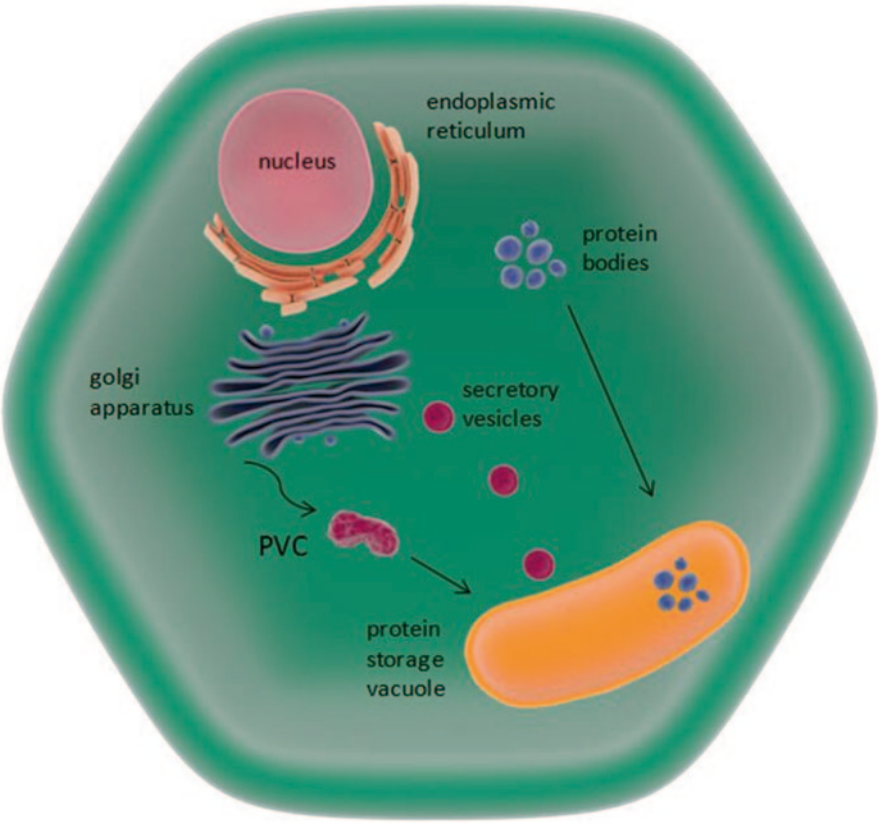

Fig. 5.2 Protein-trafficking pathways between storage organelles in seeds. ER-derived *PBs* and *PSVs* are the main target sites for the accumulation of endogenous and recombinant proteins. The trafficking of endogenous seed storage proteins follow specific and idiosyncratic routes through either the endomembrane system to PBs or PSV. In some cases, these pass through the *Golgi apparatus*, while in others, they bypass this compartment. *PVC* pre-vacuolar compartment. (Khan et al. 2012)

Advances in Seed-Based Vaccines

An important step in the development of seed-based vaccines is accumulation of high levels of the desired antigen. There are several factors that can be manipulated to control expression levels of foreign proteins in transgenic plants, such as the use of promoters, terminators, retention or signal sequences in the expression cassettes, along with optimization of coding sequences for plant expression (Chikwamba et al. 2002). Prior efforts to enhance levels of antigen expression and accumulation have demonstrated that codon bias, polyadenylation, and messenger RNA (mRNA) sequences contribute to levels of transcription and/or translation, and subsequent accumulation of a foreign antigen in plants (Piller et al. 2005). Using these tools, progress

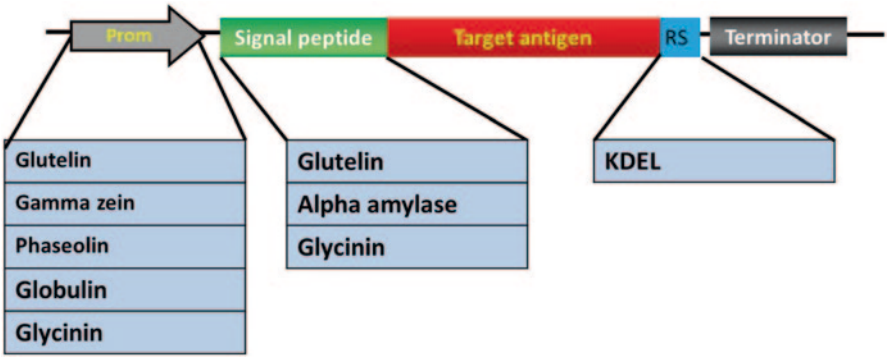

Fig. 5.3 Schematic representation of the expression cassette elements typically used to drive seed-specific expression of recombinant vaccines. *RS* retention signal

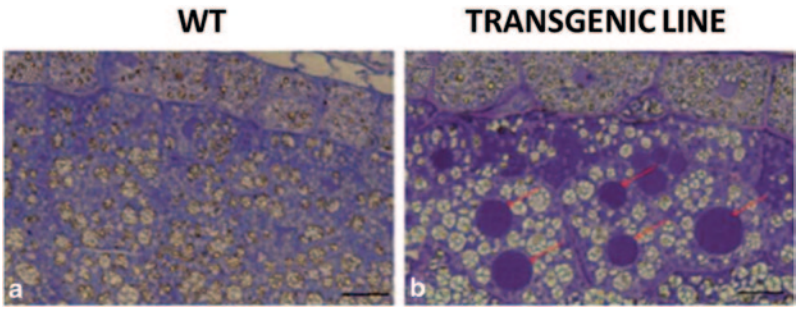

Fig. 5.4 Optical microscopy of a subaleurone layer cells in developing seeds of wild-type (*WT*) and transgenic rice plants expressing the allergen Bet v 1. Note presence of large protein bodies accumulating the recombinant protein in transgenic seeds, as indicated by the *red arrows*, compared to their absence in wild-type seeds. (Taken from Wang et al. 2013)

has been made in enhancing expression of antigens in transgenic seeds, along with promising potential of enhanced immunogenicity and immunoprotection against several diseases.

Early on, Lamphear et al. (2002) have developed transgenic lines of corn-expressing LTB of the enterotoxigenic *E. coli* (ETEC), which causes diarrhea among children as well as tourists in developing countries. Subcellular targeting of the LTB to the vacuole along with the use of a constitutive promoter has increased yield of the LTB for up to 12 % of total soluble protein (TSP). This candidate vaccine has been subsequently delivered as defatted corn germ meal to adult volunteers (Tacket et al. 2004). Seven out of nine volunteers participating in this oral feeding study have elicited an increase in serum IgG anti-heat-labile enterotoxin (LT) following vaccination. Moreover, four volunteers have shown increased levels of anti-LT IgA in their stools. This has been a pioneering first clinical trial performed using a plant-derived subunit vaccine produced in seeds. Other clinical trials have also been conducted using transgenic potato against diarrhea, *Norwalk virus,* and

hepatitis B, and promising results have been obtained (Tacket et al. 1998, 2000; Thanavala et al. 2005).

Similarly, the gene coding for LTB has also been expressed, driven by either the constitutive *Cauliflower mosaic virus* 35S (CaMV35S) or the seed-specific gamma zein promoter, in transgenic lines of maize (Chikwamba et al. 2002). Transgenic maize lines expressing LTB driven by the constitutive promoter produced LTB at levels of 0.04 and 0.01% of TSP in kernels whereas those lines expressing LTB driven by a zein promoter accumulated LTB levels reaching 0.07% of TSP in kernels. Moreover, the influence of the ER motif, SEKDEL in combination with the zein promoter in transgenic maize lines has contributed to increased LTB levels, up to 3.7% of TSP, in transgenic kernels. Furthermore, orally immunized mice with these candidate vaccines produced both serum and mucosal immune responses, while displaying reduced fluid accumulation in the mammalian gut compared to non-immunized mice.

In another study, Streatfield et al. (2001) demonstrated that targeting LTB to the ER promoted accumulation of the recombinant protein to higher levels in the presence of a targeting signal in transgenic maize. Moreover, transgenic maize seed induced an immunogenic response in BALB/c test mice similar to that of pure LTB, as demonstrated by serum levels of anti-LTB-specific IgA responses. Also, mucosal immunogenicity results showed that doses of 5 μg of LTB expressed in corn were sufficient to induce a strong mucosal IgA response, even greater than that elicited by the pure LTB. Grain of transgenic corn was also found to survive fractionation analysis, a process designed to divide seed into component parts (germ, grits, and bran). This demonstrated that antigenic proteins could survive routine grain processing, and that this formulation was enriched in particular fractions, thus offering flexibility in developing various vaccine formulations (Lamphear et al. 2002).

A similar approach was used to express LTB in soybean seeds (Moravec et al. 2007). The recombinant LTB protein expression was directed to the ER and regulated by a cotyledon-specific soybean glycinin promoter. LTB accumulated to 2.4% TSP, which is about 2 mg per seed, and did not change upon seed desiccation. Interestingly, soybean-made LTB induced both systemic IgG and mucosal IgA in immunized mice following oral administration. A remarkable finding was that optimal balance of systemic IgG/IgA immunity and mucosal immunity was achieved using a parenteral prime-oral gavage boost strategy. Also, partial protection was achieved following LT challenge of mice orally immunized with soybean-derived LTB (Fig. 5.5).

In other approach, the gene coding for the cholera toxin B subunit (CTB), driven by the endosperm-specific expression promoter glutelin *GluB-1* fused to an ER retention signal peptide, KDEL has also been expressed in rice by Nochi et al. (2007). It was observed that CTB accumulated in protein bodies of endosperm cells. Mice orally immunized with CTB-transgenic rice induced CTB-specific serum IgG and mucosal IgA antibodies with neutralizing activity. On average, CTB accumulated to levels of 30 μg per seed, and these levels remained stable and maintained immunogenicity at room temperature for more than 1.5 years. Moreover, it was reported that when present in plant biomass, CTB was protected from pepsin digestion *in vitro*.

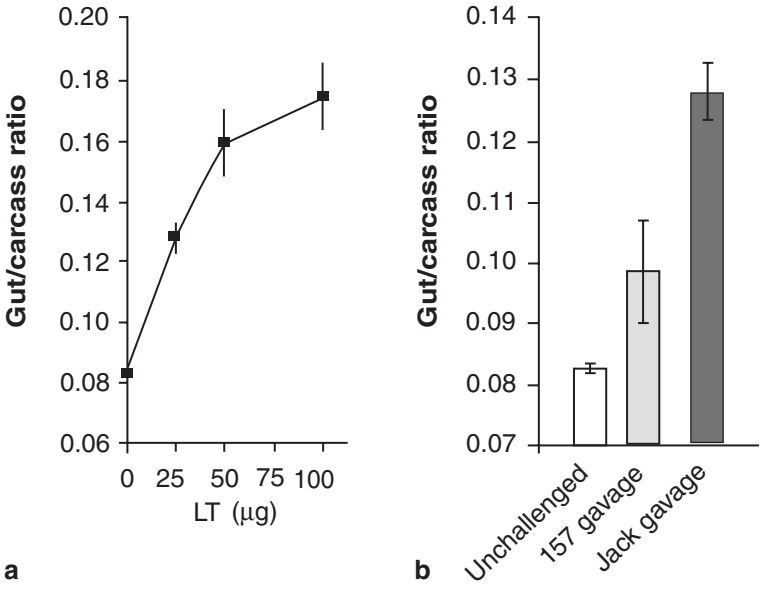

Fig. 5.5 A soybean-made LTB induces protection against toxin challenge. Test mice were subjected to immunizations at weekly intervals during a period of 48 days, and assessed for protection against a challenge with the heat labile toxin of *E. coli* on day 64. **a** Dose response curve for heat-labile enterotoxin (*LT*) in non-immunized mice. Animals were subjected to *LT* doses ranging from 10 to 100 µg. A dose of 25 µg was selected for challenge. **b** Protection observed against *LT* action. Mice orally immunized with extracts from a transgenic line expressing LTB (157 gavage group) showed reduced gut/carcass ratio when compared with a control group fed WT soybean (Jack gavage group), reflecting a substantial toxin neutralization. (Taken from Moravec et al. 2007)

In another effort for vaccine development against ETEC, expression of a K99 fimbrial subunit, FanC, has been reported in soybean. Early efforts reported that FanC levels of 0.5% TSP were detected in soybean leaves (Piller et al. 2005). Mice immunized intraperitoneally with protein extracts developed antibody titers against FanC. This was the first report on the expression and immunogenicity of an antigen in soybean (Piller et al. 2005). Subsequently, the antigenic protein was targeted to chloroplasts, and although proper targeting of FanC was observed, accumulation levels were fivefold lower than cytosol-targeted FanC. Mice intraperitoneally immunized with the protein extract developed significant antibody titers against this recombinant protein. Despite its low accumulation, it was demonstrated that chloroplast-targeted FanC was immunogenic (Garg et al. 2007).

A hepatitis B surface antigen (HBsAg) has also been used for expression in maize seeds (Hayden et al. 2012). Using an improved version of the *globulin1* promoter along with the barley alpha amylase signal sequence and a vacuolar-targeting sequence, yields of up to 0.51% TSP have been obtained. Moreover, HBsAg expressed in maize seeds has been found to be highly heat stable for a period of 1 month without degradation. Previous studies with commercialized HBsAg vaccines

have reported reduced antibody titers when the vaccine is exposed to high or even to ambient temperatures. Oil-extracted recombinant protein has demonstrated stability following hexane treatment or supercritical fluid. Following corn seed processing and temperature treatments, it has been confirmed that the grain provides a highly stable environment for the recombinant protein (Hayden et al. 2012).

Tackaberry et al. (1999) expressed the human cytomegalovirus (HCMV) glycoprotein B (gB) in seeds of transgenic tobacco. This was the first report on expression of an immunodominant antigen of the HCMV in plant seed. PSVs were identified as the main site for the deposition of the recombinant protein. Interestingly, this protein was detected in seeds that were freshly harvested as well as in seeds that had been stored for several months, thus concluding that the gB served as a plant storage protein reservoir when expressed in tobacco seeds. Further studies have reported on expression of gB in rice, with sustained expression over three generations of rice. Levels of gB were found to be both stable and immunoreactive over a period of 27 months (Wright et al. 2001; Tackaberry et al. 1999, 2003, 2008).

In other approaches, RNA interference (RNAi) suppression strategies have been used to suppress production of endogenous storage proteins expecting an enhanced antigen accumulation capacity. This was first reported by Goossens et al. (1999) in expression of arcelin in *Arabidopsis*. Later, Yuki et al. (2012) have used this technology to suppress storage proteins such as prolamins and/or glutelins in rice, thus allowing for preferred expression of the *Botulinum* neurotoxin type A antigen in the cytoplasm. They have reported a yield of approximately 100 μg protein per seed compared to levels of 10 μg protein per seed in non-silenced plants. More importantly, this candidate vaccine has induced protective immunity in mice via the intranasal route. Therefore, use of RNAi technology can aid in the development of plants that can produce a high-molecular-weight vaccine antigen with high yields.

Future Perspectives of Using Seeds as Vaccine Production Platforms

Although several antigens have been expressed in plants, there are several challenges that are yet to be overcome. Low expression levels and lack of homogeneity in expression of antigenic proteins in plants are important issues for consideration. However, several strategies have been recently developed to improve and/or maximize yield of heterologous protein production in plants. Targeting expression in seeds using seed-specific promoters along with proper signal peptides have been demonstrated to improve yields of antigenic proteins in plant-based systems. A "proof of concept" has been provided for a number of candidate vaccines, and limited clinical trials have been conducted with promising results (Streatfield et al. 2003). However, additional studies are yet to be conducted. On the other hand, the biosafety of plant bioreactors is of importance and should be addressed. High-value antigenic proteins produced in plants or plant organs should be evaluated for clinical safety and efficacy. Moreover, regulatory policies should be in place to contain gene flow in seeds to alleviate biosafety concerns.

There are a number of different strategies to prevent gene flow, such as the use of "terminator technology" which causes second generation seeds to be sterile, although this technology has been deemed highly controversial. Seeds produced with this technology will reduce the likelihood of propagation of volunteer plants and prevent escape of transgenes into non-transgenic and wild relatives. This will also prevent unintended transmission of non-food pharmaceutical products into crops destined for the food chain. Lethal factors or suicidal genes could also be useful to prevent the development of transgenic volunteers. The linkage of a lethal gene to the new trait allows for elimination of unwanted volunteer plants carrying the transgene without affecting other plants (Kuvshinov et al. 2001).

Reduction of pollen-mediated gene flow is designed to reduce gene movement, such as the modification or inhibition of flowering. Male sterility systems have also been designed, requiring the use of a pollen-specific promoter linked to a toxin gene essential for male fertility. A popular strategy is to target the transgene to genomes of organelles, such as chloroplasts, thus preventing transgene escape via pollen transfer (Hills et al. 2007). However, the complex machinery related to PBs production is outside of this compartment, therefore limiting protein processing that can take place in plastids.

In conclusion, seed-based platforms offer technological advantages to overcome some of the limitations in using plants as expression hosts for recombinant antigenic proteins and production of plant-based vaccines.

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Chapter 6
Bioreactors for Plant Biomass Production and Bioprocessing

Luz María Teresita Paz-Maldonado and José Enrique González-Ramírez

Culture Types for Plants

In vitro plant cultures can be established on defined culture media under aseptic conditions. Plant cells can be cultivated as cell suspension cultures when undifferentiated cells are used and also under different conditions as heterotrophic, mixotrophic, and photoautotrophic. Additionally, organ cultures have been successfully used, including roots and shoots, propagated in media containing hormones. Furthermore, immobilized cultures of undifferentiated tissues (callus) have also been used. This makes *in vitro* plant cultures an interesting tool for the production of different metabolites with biomedical applications such as vaccines. In this section, the main characteristics of the distinct types of plant cell cultures, including cell suspension cultures, immobilized cultures, and organ and tissue cultures, are discussed.

Cell Suspension Cultures

Plant cell suspension cultures are usually derived from stably transformed lines generated by *Agrobacterium*-mediated transformation or ballistic transformation. Callus cells from transgenic lines can be grown in a chemically defined media with regulators to establish transgenic cell suspension cultures (Rao et al. 2009). The development of an active and segregated population of plant cells in suspension cultures is dependent on the zone of collection of the plant species, the genetic background, the callus texture, and the medium/hormone combinations (Chattopadhyay et al. 2002).

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Table 6.1 Representative examples of biopharmaceuticals produced in plant cell suspension cultures

Vaccine, metabolite or protein	Plant cell platform	Application	Company	Reference
Hepatitis B surface antigen	*Glycine max* and *Nicotiana tabacum*	Protects against the hepatitis B virus	Lab scale	Smith et al. 2002
Newcastle disease virus vaccine	*Nicotiana tabacum*	Protects against Newcastle disease virus	Dow AgroSciences	Travis 2008
Human granulocyte-macrophage colony stimulating factor (hGM-CSF)	*Nicotiana tabacum*	Vaccine adjuvant in HIV-infected patients	Lab scale	Lee et al. 2004
Recombinant glucocerebrosidase	*Daucus carota*	Treatment of Gaucher's disease	Protalix Biotherapeutics	Shaaltiel et al. 2007
Paclitaxel	Taxus species	Antitumoral drug	Phyton	Arias et al. 2009
Human serum albumin	*Nicotiana tabacum*	Liver diseases	Lab scale	Sijmons et al. 1990
Human erythropoietin	*Nicotiana tabacum*	Treating anemia and myelodysplasia	Lab scale	Matsumoto et al. 1995
Human alpha-1-antitrypsin (AAT)	*Oryza sativa*	Protease inhibitor	Lab scale	Trexler et al. 2002
Outer membrane lipoprotein OspA	*Nicotiana tabacum*	Lyme disease agent	Lab scale	Navarre et al. 2006

Advantages of cell suspension culture comprise: (1) highest biomass production under optimal operational control of temperature, pH, and dissolved oxygen; (2) problems associated with the vagaries of weather, pest, soil, and gene flow in the environment are avoided; (3) primary and secondary metabolites can be produced in the exponential and stationary phase, respectively; (4) timescale needed for the production of antigens, metabolites, or proteins in plant cell cultures can be counted in days or weeks after transformation compared to months needed for the production in transgenic plants cultured in soil. Table 6.1 shows successful industrial plant cell suspension cultures used for metabolite production with biopharmaceutical applications including vaccines. These examples include hepatitis B surface antigen at laboratory scale (Smith et al. 2002) and companies like Dow Agrosciences that developed a recombinant animal vaccine against Newcastle disease virus (NDV; Travis 2008). This analysis reflects that this type of culture should be explored more extensively for producing vaccines at a large scale.

The bioreactor types that can be used for plant cell suspension cultures comprise: stirred tank, bubble column, or airlift. The latter is a better choice for minimizing shear stress. A membrane bioreactor or inclusive a flask can be used to obtain better

yields for *Oryza sativa* for foreign protein production based on plant cell suspension cultures (Huang and McDonald 2009). Stirred tank bioreactors are recommended for high cellular density cultures (20–30 kg dry weight/m^3) while airlift bioreactors are recommended for less cellular density cultures (15–20 kg dry weight/m^3; Doran 2000). Wang and Zhong (1996) have developed a bioreactor for shear-sensitive plant *in vitro* systems named the centrifugal impeller bioreactor (CIB) as an approach to obtain better yields. In order to minimize validation efforts and production costs, several disposable bioreactor designs for plant cell cultures have been developed recently (e.g., life reactor, ebb-and-flow bioreactor, plastic-lined bioreactor, wave reactors, Nestlé's wave and undertow bioreactor, and slug bubble bioreactor; Georgiev et al. 2009).

On the other hand, in this type of culture a homogeneous distribution of cells into media occurs at early stages of the culture, which is beneficial for the nutrient and oxygen transfer. However, plant cell suspension cultures present some problems such as the tendency to forming aggregates during exponential growth phase, which limits the oxygen dissolved and nutrients distribution leading to a decrease in secondary metabolite production. Additionally, rheological behavior of plant-cell suspensions tends to be non-Newtonian, compromising the momentum, mass, and heat transfer. For example, *N. tabacum* has a pseudoplastic behavior that affects the apparent viscosity. Specifically, Kato et al. (1978) indicated that the apparent viscosity of *N. tabacum* cell culture broth was increased by a factor of 27 throughout the batch culture period. A typical apparent viscosity of plant cell culture broth is 4–150 cP (0.004–0.15 N s m^{-2}). However, it is known that periodic removal of part of the culture and refilling with a fresh medium reduces the viscosity and improves the fluid flow in viscous fermentations, thus mass and heat transfer can be effective (Doran 1995).

Immobilized Cell Cultures

This type of culture is applied when favoring the plant secondary metabolism is of key importance. Some characteristics of immobilized cell culture are a lower growth flux allowing better nutrient and energy intake and contact between cell and cell, which facilitates the desired differentiation and mimics a physical–chemical plant environment. Physiological and morphological changes can also occur in this type of culture leading to a stimulation of the production and release of secondary metabolites into media, which is mediated by cellular membrane permeabilization. Therefore, product recovery is facilitated because of the existence of liquid and solid phases. Therefore, these types of cultures offer a potential for overcoming the drawbacks of plant-cell bioreactors arising from the instability of cells and the low yields of the desired compounds.

This type of culture is also named perfusion culture and the bioreactors that could be used are airlift, fluidized bed, packed bed with immobilized cells, and biofilm

Table 6.2 Examples of immobilized plant cell cultures

Metabolite or protein produce	Matrix	Plant cell	Application	Reference
AAT	Membrane	*Oryza sativa*	Prevent emphysema, hepatitis, and skin disorders	McDonald et al. 2005
hGM-CSF	Alginate beads	*Nicotiana tabacum*	Treatment of neutropenia and aplastic anemia	Bodeutsch et al. 2000
Spirostanol	Calcium alginate beads	*Solanum chrysotrichum*	Antineoplastic agent	Charlet et al. 2000
Anthocyanins	Pectin/chitosan	*Cruciata glabra*	Antioxidant and pigments	Dörnenburg 2004

bioreactors (Orozco et al. 2002). The available immobilization methods include gel entrapment, adsorption, and foam. The microencapsulation using polymeric membranes (polystyrene, nylon, polylysin-alginate) is also used with plant cells.

Advantages of immobilized cell culture comprise the following: (1) continuous operation with biocatalyst reutilization, high dilution rates; (2) immobilization of the shear stress (cells are retained in the vessel by a mechanical device such as a filter); (3) avoiding the washout effect due to a continuous operation where there is a cell feedback that increases fermentation time and then productivity; and (4) avoiding downstream processing leading to a decrease in costs.

Since secretion of secondary metabolites is a requisite when cell immobilization approaches are followed, several methods, such as temperature adjustment, electrical permeabilization, altering medium composition, and permeabilization with chemicals such as dimethyl sulphoxide, have been used to improve product recovery (Buitelaar and Tramper 1992). However, some problems of immobilized cell culture comprise the introduction of gradients in the gel beads, the necessity for product excretion, and loss of cell viability in many cases due to nutritional stress caused by a limiting mass transfer given by cell differentiation.

Immobilization techniques contribute to a considerable increase in the number of applications of plant cell cultures for the production of compounds with a high added value, such as rosmarinic acid and taxol with cancer chemotherapeutic or antioxidant properties (Hussain et al. 2012). Table 6.2 summarizes some plant immobilized cell cultures used for the production of secondary metabolites and recombinant proteins. However, this cultivation approach has not been used yet for the production of vaccines despite being suitable for the production of these biopharmaceuticals (Sajc et al. 2000).

Tissue or Organ Cultures

Tissue or organ cultures are chosen when the metabolite production requires a specialized cell-to-cell interaction and when biomass shows a high growth rate at this stage. The two main organ types for secondary metabolite and foreign protein production are shoots and roots. These can be grown in a chemically defined medium composed of inorganic salts under light and CO_2 as the energy and carbon sources, respectively. These conditions provide a unique environment that meets the requirements for foreign metabolite production such as fast cell growth, high genetic stability, and capability for producing glycosylated proteins with non-immunogenic humanized glycan patterns (Gomord et al. 2010; Gorr and Wagner 2008). Although *in vitro* cultivation of several tissues and organs has been established, the uses of these types of cultures for large-scale production of foreign proteins including vaccines are currently a challenge.

Advantages of using plant organ or tissue cultures comprise: the use of a simplest media without regulators, better yields, secondary metabolite accumulation, and highest biomass. Hairy roots in particular can be propagated indefinitely in liquid medium and retain their morphological integrity. However, in this type of cultures, transfer of oxygen and nutrients is limited, which requires an optimization of mass transfer for avoiding organ or tissue necrosis.

Studies have shown that hairy root culture has significantly improved long-term genetic and biosynthetic stability compared to suspended plant cells used for the production of foreign proteins (Sharp and Doran 2001). Shadwick and Doran (2007) used wild-type hairy root as an *in vitro* culture system for the propagation of plant viruses, suggesting a potential method for *in vitro* production of epitope vaccines and foreign proteins in hairy roots. Nevertheless, cell suspension cultures have more immediate potential for industrial application than plant tissue and organ cultures due to extensive expertise that has been amassed for submerged microbial cultures. While tissue and root cultures offer genetic stability as well as, in some instances, superior metabolic performances over suspension cultures of the cell lines, the development of appropriate bioreactors and operating techniques for these systems make it difficult to achieve a homogeneous culture environment and involve high investment and laborious experimentation (Chattopadhyay et al. 2002). Indeed, the main problems in bioreactor cultivation of hairy roots result from their tendency to form clumps inherently composed of primary roots and their bridged lateral roots, irrespective of the bioreactor type (Georgiev et al. 2007; Sung and Huang 2006). Immobilization of hairy roots by horizontal or vertical meshes as well as by cages or polyurethane foam demonstrably promotes their growth in submerged stirred bioreactors, bubble columns, airlift reactors, and drum reactors, where the roots are immersed in the culture medium (Eibl and Eibl 2002; Kim et al. 2002; Shadwick and Doran 2004). The oxygen transfer limitation can also be reduced or eliminated by growing hairy roots in gas-phase bioreactors, such as spray or droplet reactors, in which the roots are exposed to humidified air or a gas mixture and nutrients are delivered as droplets by spray nozzles or ultrasonic transducers

Table 6.3 Examples of distinct plant tissues and organ cultures used in the production of biopharmaceuticals

Vaccine, metabolite or protein	Tissue or organ cultures	Plant cell	Application	Reference
Hepatitis B surface antigen	Hairy roots	*Solanum tuberosum*	Hepatitis B vaccine	Sunil-Kumar et al. 2006
Rosmarinic acid	Roots	*Salvia miltiorrhiza*	Antioxidant	Yan et al. 2006
Artemisinin	Roots	*Artemisa annua*	Antimalarial	Patulun et al. 2007
Ginsenoside	Roots	*Panax ginseng*	Anticarcinogenic	Yu et al. 2005
Hyoscyamine	Hairy roots	*Hyoscyamus muticus*	Treatment of gastrointestinal disorders[a]	Eibl and Eibl 2008
Single chain murine IL-12	Hairy roots	*Nicotiana tabacum*	Antiproliferative	Liu et al. 2009
Vascular endothelial growth factor	Protonemal tissue	*Physcomitrella patens*	Charecterization of protonemal tissue culture	Lucumi and Posten 2006

[a] Peptic ulcers, diverticulitis, pancreatitis and cystitis

(Weathers et al. 1999). However, internal root anchor matrices are required in all circumstances in spray and mist reactors (often horizontal mesh trays and cylindrical stainless steel meshes). The most cited and largest hybrid bioreactor (bubble column-spray reactor) for growing hairy roots reported thus far is the 500 L Wilson-Bioreactor (Wilson 1997). Some examples of tissue or organ cultures are presented in Table 6.3. Eibl and Eibl (2008) reported that introduction of commercial ginsenoside production by culturing adventitious roots in 10,000 and 20,000 L balloon-type bioreactors could encourage the international development of hairy root-based manufacturing processes. Disposable bioreactors, such as wave-mixed bioreactor or temporary immersion bioreactor (TIB), are also suitable for growing hairy roots with stable morphological characteristics in a large-scale operation. Some of the bioreactors mentioned above are further discussed.

Factors Influencing Vaccine Production in Plants: Engineering Aspects

In order to achieve industrial applications on plant-based vaccine production, it is necessary to overcome the following problems: low productivity, slow growth, and genetic instability when culturing a recombinant plant. It is important to generate a cost-feasible bioprocess in vaccine production to decrease the product cost and making it economically accessible. In order to successfully cultivate the plant cells at a large scale, several engineering parameters such as cell aggregation, mixing, aeration, and shear sensitivity should be taken into account for selection of a suitable bioreactor.

Cell Aggregation

Plant cells frequently form aggregates in suspension culture, causing problems in culture rheology and in the metabolic properties of the cell (Huang et al. 2009), which may have adverse effects on plant cell growth, affecting protein yields and quality. This means that metabolite or protein productivity may be significantly influenced by the degree of cellular association and may, therefore, be affected by variations in aggregation patterns. Although moderate cell aggregation is sometimes advantageous since it enhances sedimentation rates facilitating media exchange as well as *in situ* recovery of culture broth during downstream processing, the generation of large cell aggregates (~1–2 mm) is undesirable since this complicates the bioreactor operation, enhances mass transfer limitations, and makes cell aggregates more susceptible to hydrodynamic stress resulting in cell damage (Huang et al. 2009). It is known that cell aggregation varies with cell line, culture age, method of inoculum preparation, medium composition, and bioreactor type and cultivation conditions (Huang et al. 2009). Hence, controlled aggregation of plant cells is of importance from the process engineering point of view. Therefore, culture conditions and aeration–agitation rates have to be optimized for minimizing cell aggregation and to facilitate large-scale vaccine production.

Light Irradiation

The spectral quality, intensity, and period of light irradiation may affect plant cell cultures in one way or another (Scheper and Zhong 2010). It is known that the quantitative effect of light intensity (27.2 W cm^{-2}) on anthocyanin formation by *Perilla frutescens* cell cultures favored pigment production (Scheper and Zhong 2010). Until now, a 16-h light and 8-h dark photoperiod has been reported for the production of vaccines in plant cultures (Kohl et al. 2007; Saldaña et al. 2006; Dong et al. 2005; Gu et al. 2005). In addition, a 3-mol m^{-2} s^{-1} illumination from cool white fluorescent lamps under a 16-h photoperiod has been applied to tobacco plants transformed with the urease subunit B (UreB) antigen gene from *Helicobacter pylori* (Gu et al. 2005), and a 12-h photoperiod using fluorescent lights at an intensity of 100–125 μmol $m^{-2}s^{-1}$ was used for tomato plants transformed with the gene-encoding cholera toxin B subunit (Jani et al. 2002).

Mixing

Mixing promotes better growth by enhancing the transfer of nutrients from liquid and gaseous phases to cells and the dispersion of gas bubbles. This process factor promotes homogeneity with respect to the plant cell mass and nutrients, and enhances mass and heat transfer in bioreactors.

Generally, for plant cell cultures, bioreactors can be divided into three types according to their mode of agitation: mechanically driven, pneumatically driven, or combined. Since plant cell cultures are very sensitive to mechanical stress, plant cells are therefore often grown in stirred tank bioreactors at very low agitation speeds (Chattopadhyay et al. 2002). In the literature, there are little studies using bioreactors with impellers. Huang et al. (2009) reviewed examples of plant suspension cultures, and the most used impeller was pitched blade. As fluid mixing is an important factor in the process, there is a need to investigate the effect of a constant mixing time on plant cell physiology and metabolism in long-term agitated cultures, because long mixing times decrease growth due to dead zones without mass transfer, while short mixing times increase cellular density due to a better homogenization during cultivation (Arias et al. 2009).

Aeration

Aeration of plant cell cultures fulfills three main functions: maintenance of aerobic conditions, desorption of volatile products, and removal of metabolic heat. The specific O_2 uptake rate (respiration) of plant cells depends on the cell culture line, cultivation conditions, and growth phase, but is generally of the order of 6×10^{-4} gO_2/g dry cell weight min^{-1} (Chisti 1999; Kieran 2001). The O_2 transfer rate must be sufficiently high to provide enough oxygen to comply with the respiratory demands of the cells, and therefore supporting the growth of the cells and production of desired compounds. It should be considered that both excessive and insufficient oxygen supply can hinder cell growth and secondary metabolism. In order to avoid oxygen limitation, the dissolved oxygen has to be kept above the critical oxygen concentration (Georgiev et al. 2009). For plant cells at high aeration rates, the use of the following systems is recommended: ceramic or sintering steel porous spargers, bubble-free aeration via tubes of silicone, external aeration via special devices, and oxygen enrichment.

In order to avoid low mass transfer due to foam formation, it is necessary to consider the geometry of the reactor and the use of antifoam agents is recommended (Eibl and Eibl 2002). The level of O_2 in liquid cultures can be regulated by agitation or stirring methodologies and gas flow rates, which affect bubble sizes, mixing and circulation times, gas hold-up values, and mass transfer coefficients. Airflow supply to the reactor determines the degree of aeration and agitation and also prevents settling of the plant biomass, thus influencing its growth and proliferation (Yesil-Celiktas et al. 2010).

Shear Sensitivity

Plant cells are usually sensitive to hydrodynamic stress as they have a large volume and a rigid cell wall. Shear stress reduces culture viability, cell mass, and secondary-

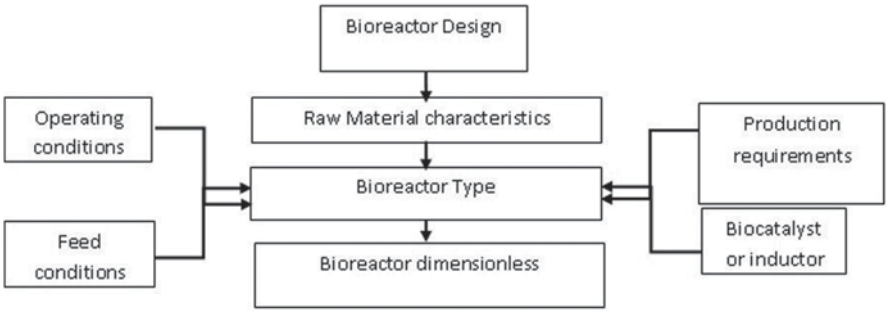


Fig. 6.1 Analysis design for bioreactor type selection and dimensions calculi. (Smith 2005)

metabolite productivity as demonstrated in cell cultures of tobacco, *Catharanthus roseus* and *P. frutescens* (Sajc et al. 2000). Reduction in cell viability (growth rate, regrowth potential, membrane integrity), release of intracellular components (variation in pH, release of proteins and secondary metabolites), change in metabolism (oxygen uptake rate, adenosine triphosphate (ATP) concentration, metabolite synthesis, cell wall composition), and changes in cell morphology and aggregation patterns were among shear-related effects identified in plant cell cultures. For cell suspensions, shear-related damage was correlated to the dissipation of energy (Kieran et al. 1995). The values reported for plant cells are generally much higher than those reported for mammalian cells (Thomas 1991), which could be explained by the presence of a thick, rigid, cellulose-based cell wall. A pitched blade turbine (axial flow pattern) with the upward-pumping mode offered advantages compared to Rushton turbines for solid suspension and for reducing shear stress to plant cells when cell damage occurs (Huang et al. 2009).

Bioreactor Design

A bioreactor design is a work of research and engineering. This work has to combine all possible parameters of relevance (raw material, product, and yield). In order to design an appropriate bioreactor for any application, several parameters have to be taken into consideration. Figure 6.1 shows a workflow for bioreactor design, including bioreactor type selection and dimensions calculi (Smith 2005).

The bioreactor type must be in accordance to production objectives. For biopharmaceuticals production, plant cell suspension cultures are the most used approach. Different bioreactor types are available for suspension cultures, all of them are discontinuous bioreactors (Eibl and Eibl 2008). These bioreactor types and design considerations are discussed in the following sections.

Table 6.4 Summary of considerations for bioreactor design to produce vaccines in plants. (Chattopadhyay et al. 2002; Huang et al. 2009; Huang and McDonald 2012)

Characteristics features of a typical plant cell	Implications for reactor design
Lower respiration rate	Lower oxygen transfer rates required
More shear sensitive	May require operation under low-shear conditions, e.g. by employing low-shear impellers and bubble free aeration
Growth as aggregates	May present mass transfer limitations that limit the availability of nutrients to cell within the aggregates
Aggregation important for secondary metabolisms	An optimal aggregate size may be required for product synthesis by manipulation of media constituents and environmental conditions
Volatile compounds (e.g. CO_2 or ethylene) may be important for cell metabolism	May need to sparge gas mixtures containing them
Product synthesis may be non-growth-associated	May require a two-step cultivation system for maximal product synthesis

Discontinuous Bioreactor

There are different configurations for this bioreactor type. The most important consideration for this device is the concept of a batch configuration. This is the most popular bioreactor type in biochemical transformations due to the process control facility, including the temperature, pH, level, and gas flow control. This bioreactor type is used to determine the maximal production level (Sajc et al. 2000). In plant cell culture applications, some critical considerations should be taken into account (Chattopadhyay et al. 2002, Eibl and Eibl 2008):

1. All cultures have to pass by a pre-preparation of charge.
2. Total homogenization should be ensured.
3. Bioreaction nature (aerobic or anaerobic bioreaction) should be considered, e.g., in plant cell cultures, a minimal oxygen concentration is required.
4. The gas feed could help on homogenization.
5. Bioreactors should facilitate instrumentation for several control levels (mainly temperature, pH, level, gas feed, and nutrient concentration).
6. Optimum aeration–agitation conditions with respect to the capacity of oxygen supply and intensity of hydrodynamic stress effects on the plant cell and tissue cultures should be considered.
7. Intensity of culture broth mixing and air-bubble dispersion.
8. Control of aggregate size (which may be important to enhance secondary metabolite production).
9. Maintenance of aseptic conditions for relatively longer cultivation period.

In addition, further considerations have to be taken into account for bioreactor design and vaccine production in plants. Some of these are shown in Table 6.4. After these considerations, it is possible to show the different bioreactor types, where several configurations are possible, but we will focus on the three principal types (Fig. 6.2):

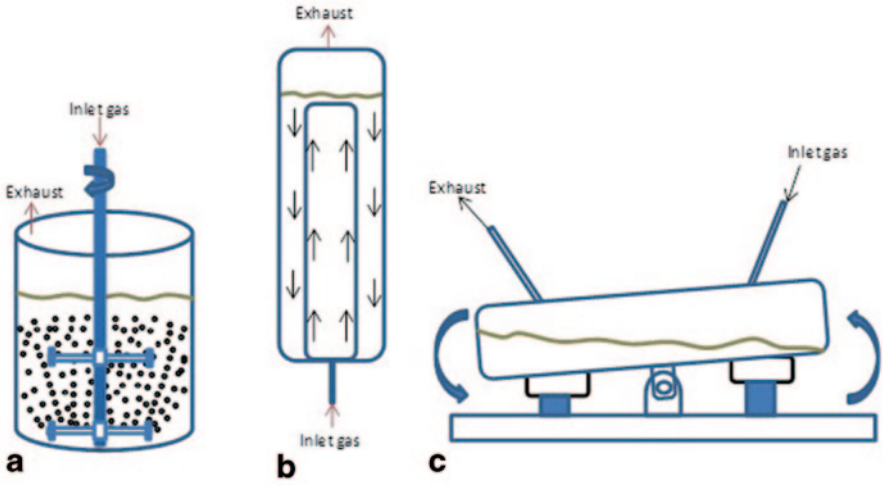


Fig. 6.2 Possible configuration for a discontinuous bioreactor for plant cell cultures **a** Total stirred bioreactor: gas flow outlet by stirrer; **b** Airlift bioreactor, arrows describe the mass displacement; **c** Wave bioreactor coupled to a mechanical agitation device

1. *Stirred tank bioreactor*. This bioreactor type consists of a vertical tank with a stirrer and it can be considered as an ideal total stirred bioreactor. Mixing and bubble dispersions are achieved by mechanical agitation, generally by a rotor coupled with baffles, which determines the mass transfer during a bioreaction (Doran 1995, Rodrigues et al. 2011). Different stirrers and baffle shapes are available in the bioprocess market and their utilization depends on the application case. The most common configuration uses a gas feed, baffles, and stirrer, in order to assure the maximal mass transfer and homogenization (Fig. 6.2a).
2. *Airlift reactor*. This reactor type belongs to the bubble column bioreactor (Sajc et al. 2000 Chattopadhyay et al. 2002). In this bioreactor, there is no mechanical agitation, and a gas flow is used as the mixing mechanism instead; thus, shear levels are lower than the stirrer reactor and mass transfer is assured by the displacement caused by the gas flow (Fig. 6.2b). This bioreactor type is less frequent than the stirrer one because air bubbles are generated, which can damage very sensitive plant suspension cells by bubble bursting.
3. *Wave reactor*. This bioreactor consists of a disposable polyethylene plastic bag enabled with circulation and inflating instruments based on airflow. This bioreactor type is considered as an emergent configuration and is typical for plant cell growth (Rodrigues et al. 2011). In this configuration, mass transfer between the medium and air is achieved by rocking the chamber back and forth (Jain and Kumar 2008). This rocking creates an agitation phenomenon that generates waves at the liquid–air interface, promoting the bulk mixing and gas transfer (Fig. 6.2c). This configuration is considered the best bioreactor type for plant cell culture suspensions (Eibl and Eibl 2008) and consequently for plant-based vaccines production.

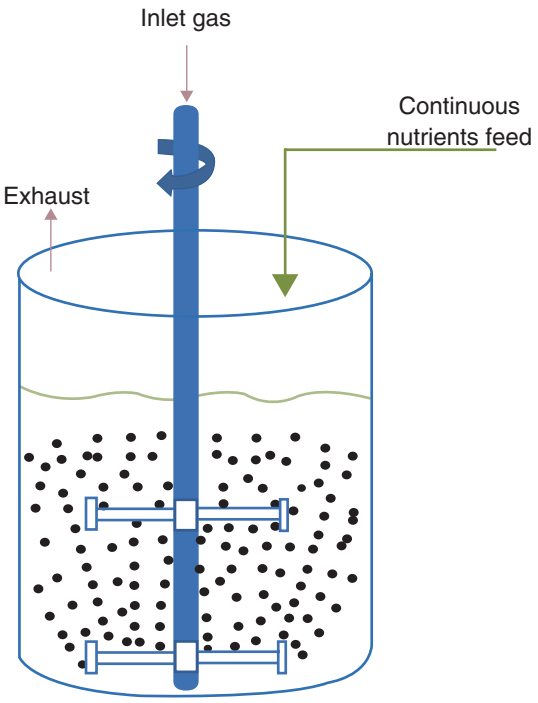


Fig. 6.3 Typical configuration of a fed-batch stirred tank bioreactor

A variation of these discontinuous bioreactors consists of fed-batch bioreactors (Fig. 6.3), where feeding the required nutrients allows for maintaining the cell growth rate at maximum levels (Mulukutla et al. 2012; Arpornwichanop and Shomchoam 2007). This strategy is very common in the bioprocess with the objective of maximizing the global production and is frequently used in the food and pharmaceutical industries (Box 6.1).

Box 6.1 A Fully Automated Plant-Based Vaccine Factory

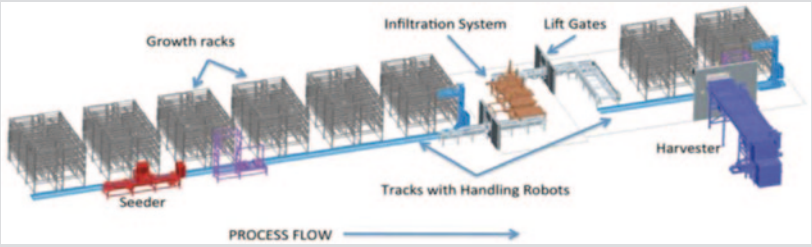
An automated process meeting the current good manufacturing practices (cGMPs) has been reported by Wirz et al. (2012) as a new convenient process for the large-scale production of vaccines. The factory is designed to be time, cost, and space efficient. It has the capacity to grow tens of thousands of plants in one batch. Plants are grown in multi-plant trays, used to handle and transport them to the different processing stations. Automation of the process relies on robots that glide up and down a track, tend the plants, and deliver trays from the lighted, irrigated growth modules to each processing station at the appropriate time.

The process consists of the following stages:

1. Seeding module. Growth tray array is populated with seeds and growth media (200 tobacco plants seeded in approximately 2 min).

2. Growth. Seeds are automatically watered and nourished for a period of several weeks. Only one seed is planted in each array location in the growth tray. A lighting system ensures that all the plants grow under the same lighting conditions. Each tray receives equivalent and controllable amounts of water.
3. Infiltration. A viral vector is transferred into the plants using a hybrid Agrobacterium/viral system. The process results in an efficient delivery of the expression vector into leaf cells, leading to an efficiency protein production. The vacuum infiltration process is accomplished while the plants are in an upside-down position, in a cycle comprising the following steps: (1) flipping trays upside down, (2) infiltration, (3) rinsing residual *Agrobacterium* solution, and (4) flipping the trays upright. The infiltration module processes trays at a rate of two trays per minute. Infiltration is carried out in a separate room in order to avoid any contamination.
4. Harvesting. Plants are allowed to grow for an additional period as the proteins are being synthesized. In this stage, plants are harvested upside down so that the cut-plant biomass can naturally fall into a collection bin. Before the trays are unloaded, they are disassembled in the harvesting module, and the hydroponic media and the remaining plant material are discarded into a waste bin for proper disposal. To reduce the burden on the waste processing system, the waste is squeezed through an auger to reduce the volume of the solid water and to separate the liquid waste. The trays are then automatically unloaded into a cart to be cleaned and sterilized for reuse.
5. Robotic transport system. Plants are milled to extract and purify the recombinant proteins using conventional procedures.

Schematic representation of the fully automated system. Taken from Wirz et al. (2012)



Another bioreactor frequently used for plant cultures is the membrane bioreactor (Fig. 6.4). This bioreactor is a compartmentalized bioreactor which is destined for *in situ* aeration, nutrient supply, or product separation by specialized membranes with specific molecular weight cutoff (MWCO). This bioreactor has the advantage of a controlled mass transfer and a low shear stress, but it has the disadvantage of a difficult scale-up (Huang and McDonald 2009).

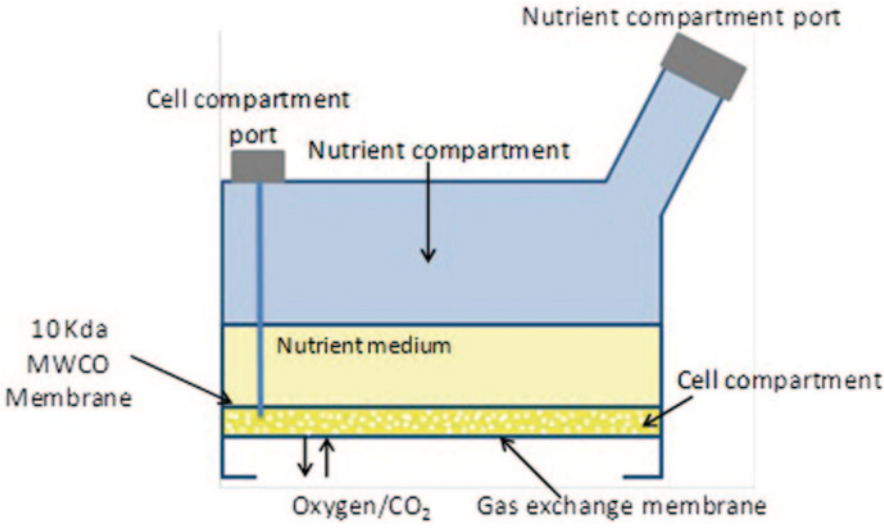


Fig. 6.4 The membrane bioreactor

Other configurations are available for cell cultures, but their use is less frequent than the mentioned above (Sajc et al. 2000). Some of these configurations are:

1. Continuous bioreactor
2. Continuous flow in a stirred tank
3. Tubular bioreactor
4. Fluidized bed
5. Packed bed

In general, these bioreactor types have advantages and disadvantages. The Table 6.5 shows the characteristics of bioreactors that are relevant for selecting the best bioreactor for vaccine production in plants.

Due to the shear sensitivity of plant cell cultures, the wave bioreactor type appears as one of the best options for this purpose. Eibl et al. (2010) presented some examples of applications for plant and animal cell cultures. In these examples, vaccine manufacture is presented as one of the most promising applications for wave bioreactors.

Raw Material Analysis and Industrial Scale-Up

Designing bioreactors requires addressing several aspects, such as the raw material characteristics and cultivation conditions. This objective is normally accomplished in an experimental study. Depending on requirements of temperature, pH, dissolved oxygen levels, expected production, shear rate, and nutrient concentration, the bioreactor configuration can be set in a laboratory device as initial approach. Table 6.6 shows some examples for the particular case of the plant cell cultures.

Table 6.5 Characteristics of the distinct bioreactor types used for plant biomass culture. (Huang et al. 2009; Huang and McDonald 2012; Eibl et al. 2010)

Bioreactor type	Advantages	Disadvantages
Stirred tank bioreactor	Commonly used Ease of scale up Useful for high viscously cell culture High oxygen mass transfer ability Good fluid mixing Alternative impellers Ease of compliance with cGMP Requirements	High shear stress around the impeller High capital and operational cost Heat generation due to mechanical mixing High energy cost due to mechanic agitation Contamination risk with mechanical seal
Air-lift bioreactor	Suitable for plant and animal cells Easy to construct and scale up Low operational cost Low contamination risk Low shear stress No heat generation from mechanical agitation Multiple-choice of internal draft tubes Good oxygen mass transfer Circulating flow pattern	Poor oxygen mass transfer ability compared with stirred-tank bioreactor Poor fluid mixing for highly viscous culture compared with stirred-tank bioreactor Serious foaming under high aeration conditions
Membrane bioreactor	Disposable equipment Ability to concentrate biomass and protein product in membrane compartment Easy to withdraw extracellular product Low shear stress Low operational cost. Difficult for on-line monitoring of culture conditions	Difficult to scale up Oxygenation required Low heat transfer rate
Wave bioreactor (disposable bioreactor)	Suitable for plant and animal cell cultures Low cell damage for agitation and aeration Low operation and capital costs Ease of compliance with cGMP Requirements Good mass and oxygen transfer Lower power consumption than the stirred tank bioreactor High flexibility, easy handling Reduced incidence of cross-contamination Savings in time and costs	Complex to scale up Scale size 1,000 L maximal Higher mixing time than the stirred tank bioreactor

It is important to underline that the bioreactor selection will provide the final production of any product. The parameters of shear rate, gas requirement (oxygen, N_2, or CO_2), temperature, agitation, and process control will improve the process and contribute to reach the maximal production. These process conditions have to be studied in laboratory scale in order to determine its influence in productivity (Kretzmer 2002; Zhong 2010).

Table 6.6 Representative examples of plant cell cultures in different bioreactor types. (Huang and McDonald 2012)

Bioreactor type	Plant species	Product	Process conditions	Expected production
Total stirred bioreactor	*Nicotiana benthamiana*	Human AAT	25 °C, 50 rpm, 40 % DO, pH 6.4	25–100 μg/L
	Oryza sativa	Human AAT	75 rpm, 27 °C, 70 % DO, 0.1–0.2 vvm	40–110 μg/L
Bubble column	*Oryza sativa*	Has	27 °C, pH 7, 1.6 vvm	74.6 μg/L
Airlift bioreactor	*Nicotiana tabacum* cv Xanthi	Single chain murine IL-12	25 °C, 0.1 vvm	14 μg/L day
Wave bioreactor	*Nicotiana tabacum* L. cv. Xanthi	Human antirabies virus mAb	25 °C	0.5 mg/L
	Hordeum vulgare	Human collagen I	22 °C, 0.5 SLPM, 9° rocking angle	2–9 μg/L

To bring to the market a product of pharmaceutical interest successfully produced in the laboratory, further process development is required. Laboratory scale consists of studying the system in flasks and small bioreactors, generally of a capacity of around 0.1–10 L. If these studies in laboratory are productive, the first consideration consists of translating it to an industrial scale with volumes of 30–1,000 L (Garcia-Ochoa and Gomez 2009; Kretzmer 2002; Vortruba and Sobotka 1992). This task is called scale-up process, where the goal is a volume augmentation to maximize productivity.

Scale-up of bioreactors is a laborious process. This transition from a laboratory device to an industrial large scale is mainly conducted by meeting the following points:

1. Cost effective
2. High reliability
3. High cell density and viability
4. High product quality
5. Easy product recovery
6. High yield
7. High safety for personnel
8. Proper control process

Since all these parameters will determine the best strategy to accomplish a good scaling-up, several considerations about the above mentioned factors have been reviewed. Figure 6.5 shows strategies for industrial-scale bioreactors designed as

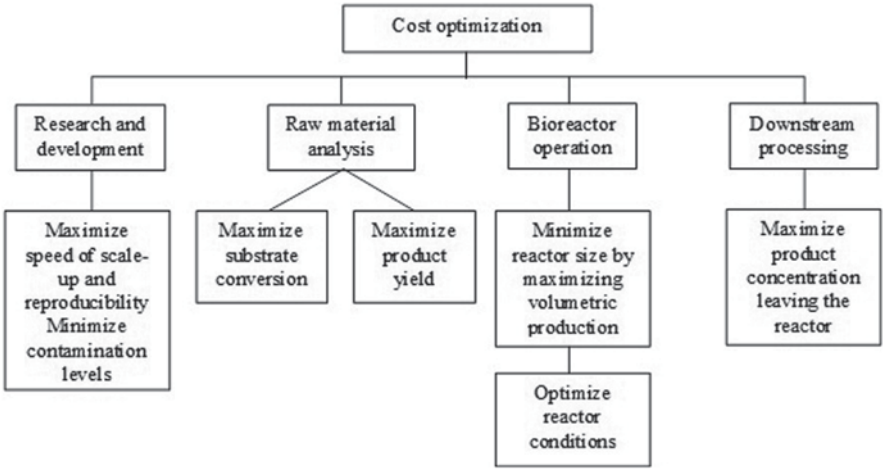


Fig. 6.5 Strategies for industrial scale bioreactors design as function of optimization cost. (Doran 1995)

function of cost optimization. It is evident that all optimization for costs must consist of maximizing and minimizing several factors, i.e., in minimizing, the product yield operating cost will decrease. Zuber et al. (1997) describes several parameters to calculate the cost of the scale-up in bioreactors.

Further considerations have to be studied, such as governing production restrictions, shear stress, adequate mixing, hydrodynamics, and mass transfer between the liquid and gas phases (Zhong 2010; Garcia-Ochoa and Gomez 2009; Acai and Polakovic 2007), geometry, kinetics (Znad et al. 2004), and medium cost (Votruba and Sobotka 1992). Baart et al. (2007) presents a methodology to scale up a bioreactor for the vaccine production. They describe mathematically all the parameters used to scale up as mentioned above. Furthermore, in order to scale up, different methods based on constant process variables (Oosterhuis 1984) can be used, which include the following:

1. Constant power/volume (P/V)
2. Constant $k_l a$ (mass transfer coefficient)
3. Scale-up based on constant tip speed
4. Equal mixing times
5. Combination of different operating variables

Meeting all the requirements mentioned above have lead to the necessity of designing alternative bioreactor configurations, particularly those that avoid or reduce shear at the large scale. Control of plant morphogenesis, biomass growth, and process control parameters, such as pH, temperature, O_2, and CO_2 levels and others, could represent a difficulty for a large-scale bioreactor (Yesil-Celiktas et al. 2010). Further consideration and other scale-up methods may also be considered. Takayama (1991) defines the liquid culture method as the best method to scale up a bioreactor for plant cell cultures. This method allows for designing a bioreactor of up to 20 m^3.

Optimization of Process Parameters

There are a variety of optimization methodologies in order to optimize a bioprocess. The response surface methodology (RSM) is presented as one of the best approaches to optimize a bioprocess. It is considered a promising tool for optimization of media in plants and tissue cultures. It helps to eliminate the less significant parameters and studying a large number of factors with only a few number of experiments with combined effect of all the factors involved, which is not possible in conventional single-variable optimization (Prakash et al. 2002). Some applications of this methodology have been reported by Omar et al. (2004) with a study of growth medium for *Centella asiatica* cell culture, and Naveenchandra et al. (2011) who have used the RSM in order to optimize the culture media for *in vitro* shoot development of *Solanum melongena* L. for micropropagation, and Niedz et al. (2007), who studied the *Brugmansia x candida* shoot multiplication and sweet orange non-embryogenic callus growth. In general, the RSM has demonstrated its utility in the optimization process. In the next sections, this methodology is described and analyzed in order to present all the possibilities and procedures implied on it.

The Response Surface Methodology

Since bioreactors are different according to its purpose, the process optimization of a bioprocess will depend on the nature of the culture. Normally, this nature determines the range of operational conditions for cell growth. Some examples of these operational conditions are: temperature, gas flow (oxygen or an inert gas as nitrogen or carbon dioxide), pH, liquid level, dissolved oxygen in medium, concentration, etc. (Doran 1995). Thus, the objective consists of finding the best operational conditions in order to accomplish bioprocess optimization. A frequently used strategy on bioprocess optimization is the use of RSM. RSM was developed by Box and Wilson in 1951 to aid in the improvement of manufacturing processes in the chemical industry. The purpose was to optimize chemical reactions to obtain, for example, high yield and purity at low costs. This was accomplished through the use of sequential experimentation involving factors such as temperature, pressure, duration of reaction, and proportion of reactants. The same methodology can be used for modeling or optimizing any response that is affected by the levels of one or more quantitative factors.

The general scenario is as follows: The response is a quantitative continuous variable (e.g., yield, purity, cost), the mean response is a smooth but unknown function of the levels of p factors (e.g., temperature, pH), and the levels are real-valued and accurately controllable. The mean response, when plotted as a function of the treatment combinations, is a surface in $p+1$ dimensions, called the response surface (Dean and Voss 1999). This response surface can be represented as a 3D plot or a contour plot (Fig. 6.6).

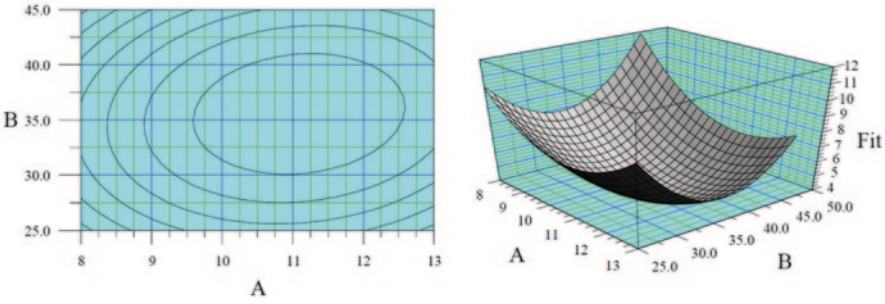


Fig. 6.6 Hypothetical surface response. *Left* contour plot; *right* 3D plot

The following example illustrates the RSM. Imagine the growth plant process, which is affected by a certain amount of water x_1 and sunshine x_2. The plant can grow under any combination of treatment x_1 and x_2. Therefore, water and sunshine can vary continuously. When treatments consist of a continuous range of values, an RSM is useful for developing, improving, and optimizing the response variable. In this case, the plant growth y is the response variable, and it is a function of water and sunshine (Bradley 2007). It can be expressed as

$$y = f(x_1, x_2) + e \tag{1}$$

The variables x_1 and x_2 are independent variables where the response y depends on them. The dependent variable y is a function of x_1, x_2, and the experimental error term denoted as e. The error term e represents any measurement error on the response, as well as other type of variations not counted in f. In most RSM problems, the true response function f is unknown. In order to develop a proper approximation for f, the experimenter usually starts with a low-order polynomial in a small region. If the response can be defined by a linear function of independent variables, then the approximating function is a first-order model. A first-order model with two independent variables can be expressed as:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + e \tag{2}$$

If there is a curvature in the response surface, then a higher-degree polynomial should be used. The approximating function with two variables is called a second-order model:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{12} x_1 x_2 + e \tag{3}$$

In general, RSM applies one or the mixture of both of the above models depending on factor number and experimental design. In each model, the levels of each factor are independent of the levels of other factors. In order to get the most efficient result in the approximation of polynomials, the proper experimental design must

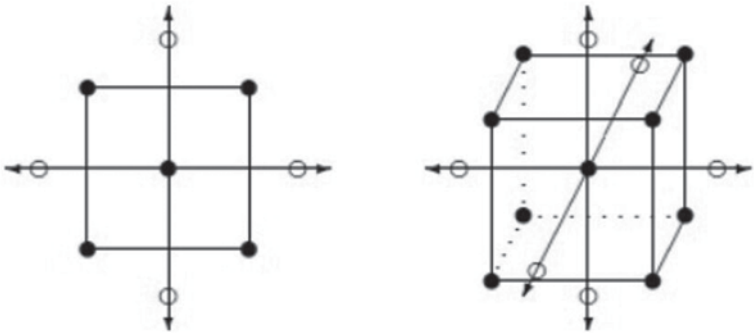


Fig. 6.7 General representation of the central composite design. *Left* $p=2$; *right* $p=3$

be used to collect data. Once the data are collected, the method of least squares is used to estimate the parameters in the polynomials. The response surface analysis is performed by using the fitted surface. The response surface designs are types of designs for fitting response surface. Therefore, the objective of studying RSM can be accomplished by:

1. Understanding the topography of the response surface (local maximum, local minimum, ridge lines).
2. Finding the region where the optimal response occurs. The goal is to move rapidly and efficiently along a path to get to a maximum or minimum response so that the response is optimized.

In order to carry out the optimization by RSM, an experimental design is needed. The best experimental design for this optimization technology is the central composite design. This design consists of a standard first-order design with n_f orthogonal factorial points and n_0 center points, augmented by n_a "axial points."

The convention of coding the factor levels is followed so the factorial points have coded levels ± 1 for each factor. However, it should be noted that some software packages, including Statistical Analysis System (SAS), Minitab, and Design Expert will recode the levels in a central composite design before doing the analysis. Under this convention, axial points are points located at a specified distance α from the design center in each direction on each axis defined by the coded factor levels. The most popular experimental designs are based on two or three factors (defined by p variables). The objective of this experimental design is exploring all the possible points in the process. Since coded levels ± 1 are defined as first-order models, the axial points α are developed as a second-order model (Dean and Voss 1999). Figure 6.7 shows an example of a central composite design for $p=2$ and $p=3$.

It is important to underline that the central composite design has to be in the region of process operability and it works in a region of interest that depends on the optimization objectives (Fig. 6.8). This experimental design, then, requires a previous process characterization.

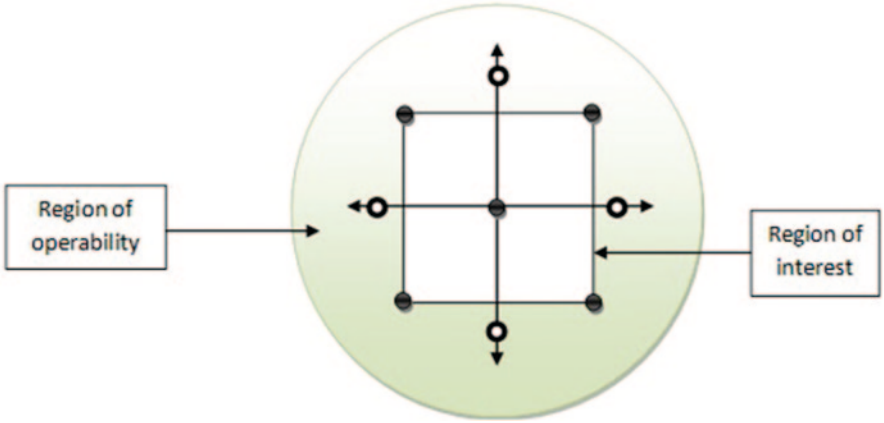


Fig. 6.8 Operability and interest regions for a central composite design

Then, the central composite design is based on the 2^p experimental designs. This means that the minimal number of experiments is defined by the next equation:

$$N_{\text{exp}} = 2^p \tag{4}$$

where N_{exp} is the number of experiments and p is the factor number, and thus, this arrangement is called factorial. This number of experiments is enough to build a first-order model.

Once this minimal number of experiments is defined, experimental error e can be calculated by n repetitions of the central point; then, the equation is transformed as follows:

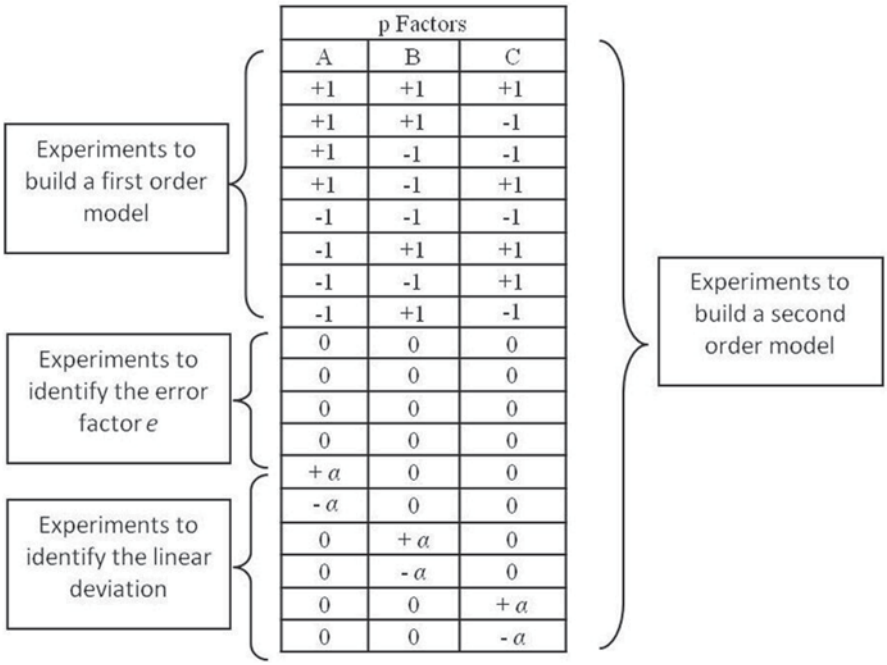
$$N_{\text{exp}} = 2^p + n \tag{5}$$

Finally, axial points α can be used to identify the linear deviation of the response. This deviation can be represented by two axial points for each p factor as follows:

$$N_{\text{exp}} = 2^p + 2p + n \tag{6}$$

In a real sense, this is the maximal number of experiments for the central composite design and is also called the "complete central composite design." This maximal number of experiments is used only if the objective is to build a second-order model (Khuri and Mukhopadhyay 2010). The axial points α value is calculated by the number of experiments in the factorial section as follows:

$$\alpha = \left(2^p\right)^{1/4} \tag{7}$$



p Factors		
A	B	C
+1	+1	+1
+1	+1	-1
+1	-1	-1
+1	-1	+1
-1	-1	-1
-1	+1	+1
-1	-1	+1
-1	+1	-1
0	0	0
0	0	0
0	0	0
0	0	0
$+\alpha$	0	0
$-\alpha$	0	0
0	$+\alpha$	0
0	$-\alpha$	0
0	0	$+\alpha$
0	0	$-\alpha$

Fig. 6.9 Central composite design for p = 3 ± 1 define de coded values for any p factor to all the possible combination; 0 Define de central points; ± α represents axial points

Figure 6.9 presents the central composite design for $p = 3$. This design permits to build an empirical model (first- or second-order model). The model is optimized by the variance analysis called ANOVA, which is a statistical method used to fit the model parameters using the response values. Selecting the statistically significant parameters allows for building the contour or 3D surface responses in order to study the influence of the factors in the process. Once the model is optimized and the contour or 3D figures plotted, the process can be optimized by searching the minimal or maximal points that depends on the optimization objective. Figure 6.10 shows the whole process.

Control in Bioreactors

The control system is one of the most difficult tasks in operating a bioreactor. In order to design a control boucle adapted to a bioreactor, a list of devices is needed. This list could be summarized as follows:

1. Instrumentation
2. Analytical techniques

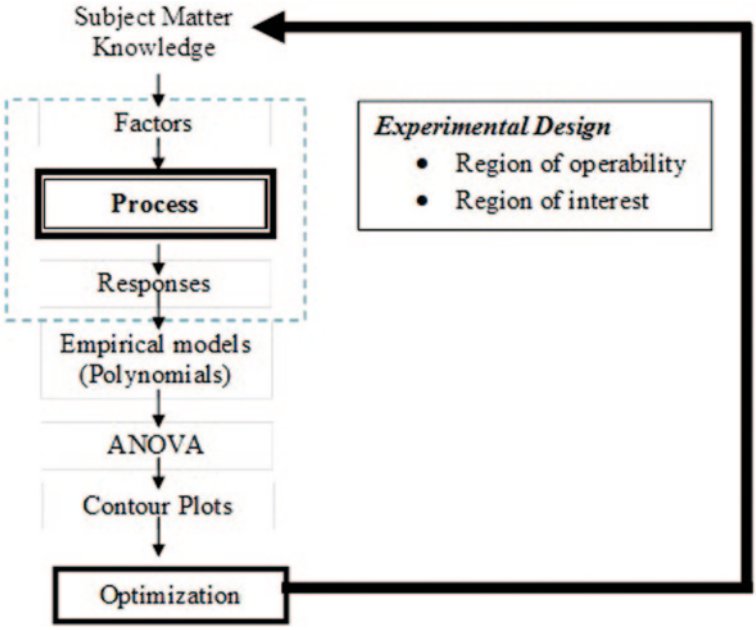


Fig. 6.10 Overview of the optimization process by the RSM

3. Monitoring
4. Process characterization
5. Mathematical model
6. Control loop

In order to operate a bioreactor, several instruments for measuring the operation conditions and results are needed. These operation conditions or response processes can be physical, chemical, or biological properties. Some of these properties are listed in Table 6.7.

Typical instrumentation of a bioreactor consists of installing *in situ* sensors to measuring and monitoring some of the physical and chemical properties, which are listed in Table 6.8. Sensors used in bioprocesses have to be able to measure the interest region of the property, having a high accuracy.

Distinct technologies have been developed for monitoring the plant growth in bioreactors. Some of these technologies, based on online or off-line analysis techniques, are related mainly to the cell growth or to physiological status, such as the biomass concentration, nutrient consumption, oxygen uptake rate (OUR), carbon dioxide production rate (CPR), and respiratory quotient (RQ). In order to estimate the cell concentration, techniques used for monitoring this parameter are based on the changes in medium conductivity, osmolarity, dielectric properties, and culture turbidity. One of the most recent online techniques to characterize plant suspension cultures is the focused beam reflectance measurements method (FBRM), the

Table 6.7 Physical, chemicals and biological properties of relevance on bioreactor monitoring. (Doran 1995)

Property	Observations
Physical	
Temperature	Online sensor, easy to install, low cost
Pressure	Online sensor, easy to install, high cost for high precision instrument
Reactor weight	Online instrument, difficulty to install
Liquid level	Online sensor, easy to install, low cost
Agitator speed	Online sensor, easy to install, medium cost
Power consumption	Online sensor, difficulty to install, high cost
Gas flow rates	Online sensor, pressure requirements, high cost
Medium flow rate	Online sensor, easy to install, medium cost
Culture viscosity	Online sensor, indirect measure, high cost
Chemical	
pH	Online sensor, easy to install, low cost
Dissolved O_2	Online sensor, high sensibility, high cost
Dissolved CO_2	Online sensor, high sensibility, high cost
Exit gas composition	Online sensor, high cost
Conductivity	Online sensor, easy to install, low cost
Broth composition	Online sensor, high cost
Biological	
Biomass concentrations	Online sensor, high cost
Enzyme concentrations	Offline technique analysis, high cost
Biomass	Online sensor, high cost
DNA, RNA, protein	Offline technique analysis, high cost
ATP/ADP/AMP, NAD/NADH	Offline technique analysis, high cost
Morphology	Offline technique analysis, high cost

Table 6.8 *In situ* sensors available for bioreactors. (Nielsen 1999; Creus-Solé 1997)

Culture parameter	Sensor type	Range of measure	Accuracy
Temperature	Pt-100	0—150 °C	0.1 °C
	RTD	0—150 °C	0.5 °C
Pressure	Piezoresistor	0—2 bar	20 mbar
Gas flow	Thermal mass flow rate	0—20 $Lmin^{-1}$	0—20 mL min^{-1}
	Electromagnetic valve	0—20 $Lmin^{-1}$	±1 %
pH	pH electrode	2—14	0.02
pO_2	Polarografic clark electrode	0—400 mbar	2 mbar
pCO_2	Membrane covered pH electrode	0—100 mbar	2 mbar
Level	Radar	0—30 m	2.5 mm
	Radiation	0—2.5 m	±2 %
	Lasser	0—2 m	±2 %
Viscosity	Ultrasound	0—50,000 cp	–
	Torque element	0—300,000 cp	±1 %

Table 6.9 Summary of advantages and limitations for distinct in-line biomass sensors. (Nielsen 1999)

Principle of detection	Characteristics
Optical density	Easy to interpret (wide linear range) It generates some interferences
Culture fluorescence	Good for monitoring little changes in biomass concentration Sensor with a high sensitivity It generates a lot of interferences
Capacitance	It is sensible to the cellular activity Good for high biomass concentrations Air and agitation generate interference Difficulty to interpret results
Ultrasound	It is sensible to the cellular activity Wide linear range Easy and self-cleaning Air and agitation generate interference Difficulty to interpret results It is sensible to temperature changes

sensors built with metal oxide semiconductor, and the fiber-optic probe (Huang and McDonald 2009). Table 6.9 shows some of the principles that could be used for monitoring as well as their advantages and limitations.

Control Strategies

Bioprocess control is a complicated subject as many variables have to be kept under control. The objective of a control system is maintaining certain variables constant in quantity or quality. Thus, there are many variables that can change (temperature, pressure, pH, etc.) and may alter other variables such as the biomass production or could activate the production of non-desirable chemical or biochemical species. Then, the objective of a control system consists of maintaining variables constant such as temperature or pH (that can be altered due to external disturbances) in order to reach a constant biomass rate production or blocking other undesired bioreactions.

A system control comprises several components (Fig. 6.11). The first element is a sensor (e.g., thermocouple, temperature sensor) that is connected to a transmitter, which sends an electronic signal to the controller (the second element). This controller subsequently receives the measurement that is compared with a desired value or set point. The comparison between the temperature measurement and the set point produces an activation of this controller that decides what proceeds in order to maintain the temperature at the desired value. This element takes the best decision to control a variable in a constant desired value and its last task is sending a signal to the final control element or actuator, which in turn manipulates another device, such as a control valve, a variable speed motor or pump or a conveyor, to

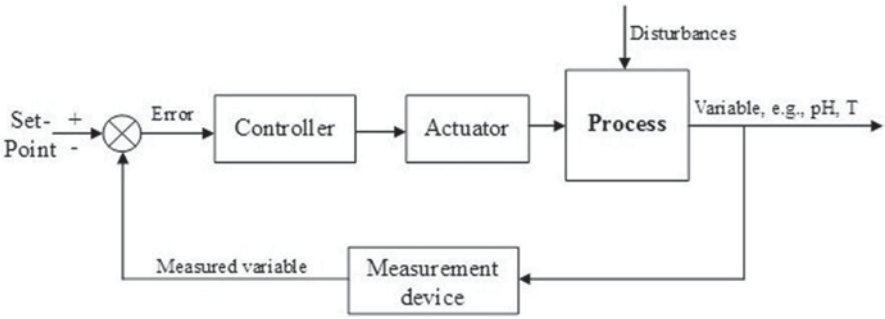


Fig. 6.11 A feed-back control strategy

close the control loop. This is a control strategy known as the feedback control, the most common in bioprocesses (Smith and Corripio 1997).

This feedback control strategy is carried out for every variable of the process; thus, the control loop will need several measure instruments and several control loops interconnected. Therefore, a typical control system for a bioreactor will need a feedback control for temperature, substrate feed, pH, dissolved oxygen, or carbon dioxide, all of them interconnected in a process monitoring (Fig. 6.12).

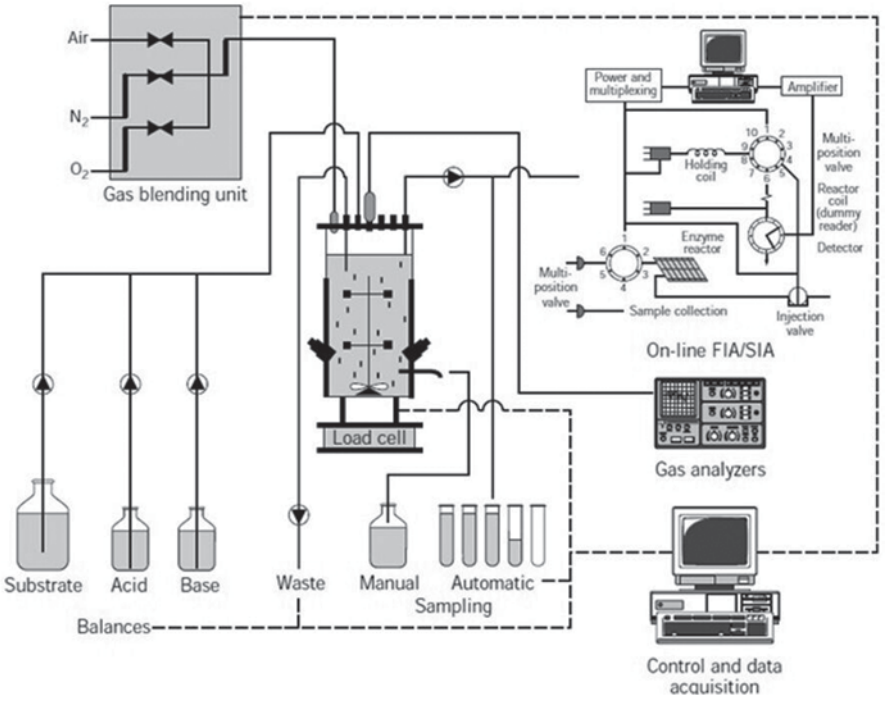


Fig. 6.12 Typical bioreactor instrumentation and control flow injection analysis (FIA) and sequential injection analysis (SIA). (Nielsen 1999)

A bioreactor system is known to show a non-linear behavior. For this reason, different advances have been developed for several applications during a biotransformation. Strategies such as the fuzzy logic, proportional–integral–derivative (PID), artificial neural network, and predicting by modeling control or combinations of them can be found in the literature. Some examples of these advances are showed in Table 6.10.

Approaches for Downstream Processing

Bioprocessing in the production of plant-derived vaccines comprise several well-established techniques. In general, these techniques can be devised in four steps:

- To separate impurities from the raw material
- To concentrate solution
- To capture product
- To capture contaminant traces

The unitary operations conducted to address this aim can be categorized as batch or continuous operations. To determine the concept in batch or continuous operations depends on the available technology and the process plant capacity. It is estimated that a production higher than 10,000 T/year requires processes and separation techniques in a continuous manner. In the case of processes using raw materials from agriculture for the manufacture of high-purity products, the process is considered as a batch (Smith 2005). Due to these criteria, vaccine production and downstream processing are preferably approached as batch processes. Table 6.11 shows the most frequent unit operations performed to purify product from plant biomass.

Further considerations could be applied for vaccine production in plants. The first one consists of defining the vaccine delivery route. The simpler case is oral delivery, where no purification of the antigen is needed when edible plant biomass is involved. In this case, lyophilization process can be applied to guarantee stability at room temperature and allowing for homogenization and proper dosage. The lyophilization process consists of eliminating water content in the plant biomass by a freeze-dried process. This process has various advantages that could be used for the manufacturing vaccines from plants, such as a long-term storage, increase of therapeutic protein content, antigen stability, and decrease of microbial contamination. Two different ways can be employed to deliver the vaccine: as a dry powder that is resuspended in water right before administration or as a gelatin-encapsulated product for direct oral administration (Sala et al. 2003; Kwon et al. 2012). Figure 6.13 explains the vaccine preparation.

When parental formulations are pursued, purification steps must be performed in order to obtain a highly pure recombinant antigen. In this case, conventional purification strategies can be applied following good manufacturing practices

Table 6.10 Control strategies in bioprocess transformations

Control strategy	Objective	Observations	Reference
ANN-MPC	To design a controller for a light-algae bioreactor during the continuous cultivation of *Spirulina platensis*	The controller can intelligently learn the complicated dynamic performances and automatically, robustly and self-adaptively regulate the light intensity illuminating on the bioreactor	Hu et al. 2012
MPC	To control a substrate concentration at a desired condition in a fed-batch fermenter	This work addresses an optimization-based control strategy for a fed-batch bioreactor where an ethanol fermentation process is chosen as a case study	Arpornwichanop and Shomchoam 2009
MPC-PID	To develop the mathematical model for a bioreactor functioning at multiple operating regions	Adaptive controller for several applications	Rajinikanth and Latha 2012
ANN-MPC	To use feedforward neural networks for dynamic modeling and temperature control of a continuous yeast fermentation bioreactor	It has been designed a model process control by the ANN. The methodology has been compared with a PID control system	Nagy 2007
MPC-GA	To integrate a genetic algorithm to a dynamic model process for an optimal control.	They introduce a few filters into a real coded genetic algorithm as additional operators.	Sarkar and Modak 2004
PID-GA	To design a feedforward feedback (PID) controller for control of glucose concentration during the *E. coli* fed-batch cultivation process	The controller maintains glucose concentration at a desired set point. An equation for correction of the measured glucose based on Kalman filter estimates biomass concentration and bacteria growth rate	Slavov and Roeva 2012

ANN artificial neural network, *MPC* model predictive control, *PID* proportional, integral and derivative control, *GA* genetic algorithm

Table 6.11 Unit operations in downstream for plant cell culture suspensions. (Sacj et al. 2000; Xu et al. 2011)

Separation step	Unit operation	Observations
Eliminating impurities/ raw materials preparation	Centrifugation	Batch process
	Filtration	Batch or continuous process
	Cell disruption with ultra-sonication	Batch process
Concentrating solution	Ultrafiltration	Batch process Non-selective process
	Precipitation	High process time Non-selective process
	Liquid-liquid extraction	Batch or continuous process Secondary process to purify solution
	Adsorption	Batch process Selective process
Recuperating product	Chromatography	Molecular size Charge Hydrophobicity Molecular recognition
Polishing	Affinity chromatography	High specificity and sensitivity are required

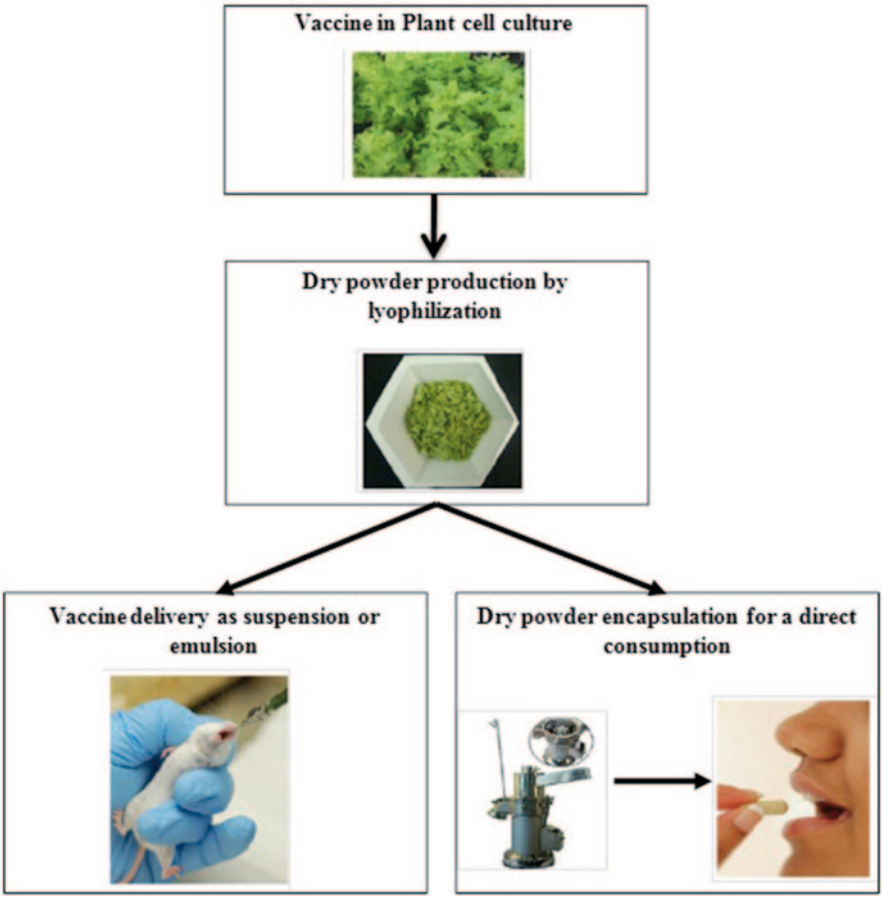


Fig. 6.13 Vaccine's preparation methodology for an oral delivery. (Xu et al. 2011)

(GMPs). For example, immobilized metal affinity chromatography (IMAC) is considered an option to purify antigenic proteins from plant cell culture (Malabadi et al. 2011). Hamorsky et al. (2013) have been applied the IMAC technology to purify the cholera toxin B subunit protein (CTB) from leaves when expressed in *Nicotiana* plants. They investigate a possible rapid and scalable plant-based production of a CTB in mass vaccination against cholera outbreaks. Buyel et al. (2012) have also applied IMAC technology for the purification of a vaccine against human papillomavirus. These purification approaches are specially required when transient agroinfiltration-based expression approaches are followed, as bacterial contaminants such as lipopolysaccharides (LPS) are present in the plant biomass and the typical expression hosts contain toxin plant compounds.

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Chapter 7
Plant-Based Vaccines Against Influenza

Andrea Romero-Maldonado, Jorge A. Salazar-González and Sergio Rosales-Mendoza

Introduction

The influenza virus is a globally important respiratory pathogen that causes a high degree of morbidity and mortality with the capacity of evolving into a pandemic behavior. Challenges in reaching an appropriate immune coverage against influenza are posed by the high viral mutation, which leads to changes in the antigenic determinants affecting the recognition of the main neutralization target surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA; Nichol and Treanor 2006; Watanabe et al. 2012; CDC 2013). It is estimated that the generation of one to two mutants occur each year (Webster et al. 1992). Infants represent the most susceptible age group for this infection, but the risks of complications, hospitalizations, and deaths due to influenza are higher among persons aged 65 years and older, young children, and persons having certain medical conditions (CDC 2013).

The family *Orthomyxoviridae* is composed of RNA viruses that infect vertebrate animals and comprises six genera, influenza A, B, and C viruses being the most prominent. These are distinguished on the basis of the sequence of internal nucleoprotein and matrix proteins. Influenza A viruses are naturally able to infect a range of animal species and are further categorized into subtypes determined by the antigenicity of HA and NA. Each influenza strain derives its name from the subtype of surface glycoproteins (Choi et al. 2013). Antigenic properties of influenza variants are given by HA and NA (Oxford 2000). HA mediates the viral attachment into host cells and fusion of membranes, which leads to viral entry and the beginning of the virus replication cycle. HA is translated as a single protein and subsequently processed by a host protease into the HA1 and HA2 proteins. The amino acids at

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the cleavage site are important in determining the virulence of the virus, which becomes highly virulent if these amino acids are lipophilic (Skehel and Wiley 2000).

Immune responses elicited by HA increase host resistance to influenza and reduce the recurrence and severity of the infection (Clements et al. 1986). However, newly emerging influenza viruses overcome such protection as they contain antigenic variations which comprise antigenic drift and shift (Ping et al. 2008). Antigenic drift consists of minor changes in the HA and/or NA sequences, e.g., amino acid substitution, leading to antigenic change. However, antigenic shift consists of the generation of new virus subtypes possessing a mixed HA and NA from different subtypes. Selection pressure in the environment influences on which HA variations prevail (e.g., regions implicated on adaptive evolution or responsible for antigenicity variation). In contrast, changes in the glycosylation patterns do not have a crucial influence in the antigenic properties (Blackburne et al. 2008).

Conventional influenza vaccines consist of inactivated virus previously propagated in chick embryos or cell cultures. This is a widely used system for many types of vaccines currently available in the market, but high reactogenicity is one of the risks associated with these vaccines (WHO 2006). Another limitation is given by the fact that some viruses elected for vaccine production exhibit a low replication rate leading to poor yields (Lu et al. 2005; Gregersen et al. 2011). Egg-based virus culture depends on embryo supply that may limit the production and must be performed under high biosafety conditions and specific procedures of waste disposal since a pathogenic agent is involved in the process. Production cost for a single dose of influenza egg-based vaccine is approximately US$ 1, representing a high cost, considering that the process is focused on the production of a single type of seasonal vaccine requiring an investment that would be difficult to afford by developing countries. Therefore, this conventional system is fairly improvable in terms of costs, robustness, and safety (WHO 2006).

The Use of Plants as Next-Generation Platform for Manufacturing Influenza Vaccines

Subunit vaccines consist of individual immunogenic proteins that are intended as immunoprotective antigens, providing a safer approach than immunizing with the whole inactivated pathogen. In this field, the use of plants as a production platform offers several advantages. This approach can be scaled up easily in greenhouse-based manufacturing facilities to meet global health-care needs and the cost of plant biomass is fairly less than eggs. Plants possess an outstanding capacity of efficiently producing complex proteins at high yields and low costs. During the past decade, plant-based platforms have been adopted for the production of influenza subunit vaccines, mainly in the modality of virus-like particles (VLPs), which represent a highly efficient subunit vaccine modality. VLPs consist of protein shells that resemble a virus, allowing them to be efficiently recognized by the immune system, but

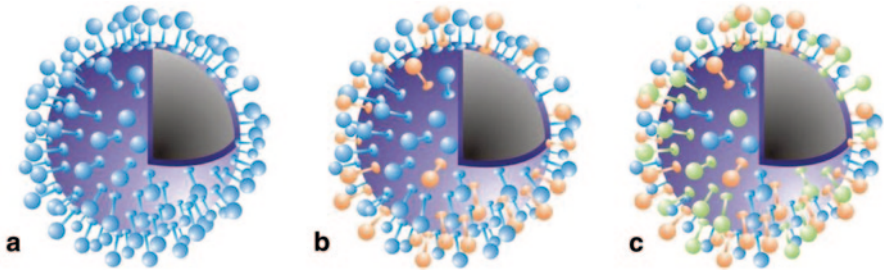

Fig. 7.1 Schematic representation of the distinct virus like particles (*VLPs*) produced in plants as a convenient immunogens for vaccination against influenza. **a** VLPs comprising hemagglutinin (*HA*) (*blue spikes*). **b** VLPs comprising HA and neuraminidase (*NA*) (*orange spikes*). **c** VLPs comprising HA, NA, and M1 (*green spikes*). Envelop is formed by the membrane from the host

lacking the replicative mechanisms. The use of VLPs in vaccination has multiple advantages as they can elicit potent immune stimulation at low doses and provide immunological memory. VLP-based vaccines have also provided protection against different strains of viruses other than those used for vaccine formulation (D'Aoust et al. 2008; Medicago Inc. 2013). This chapter will elaborate on advanced cases of plant-based influenza vaccines, most of them based on VLPs assembled with distinct influenza virus proteins (Fig. 7.1).

Plant-Based Vaccines Developed Against Influenza

In general terms, transient expression platforms have been adopted as the main approach for producing plant-based vaccination models against influenza. This approach allows for the expression of a particular antigen in a short period of time; thus, it is proposed that pandemic strains can be targeted shortly. Chapter 3 provides a detailed analysis of the principles of these expression systems. A number of unique advantages can be identified in this expression modality: A period of few weeks is required to achieve the production of a new immunogen derived from a specific seasonal strain, polyvalent vaccines can be produced by merely co-expressing antigenic proteins from distinct strains in a single transformation procedure, very high yields of protein are achieved, and manufacturing can be performed in full containment which facilitates meeting regulatory requirements.

Table 7.1 presents a comprehensive summary of the developments in the field of plant-based vaccines reported thus far. Biotechnological industries are interested in developing influenza vaccines using plant-based approaches; thus, several efforts have been conducted in this field during the past decade.

The Fraunhofer Center of Molecular Biotechnology (USA) has reached several achievements and insights on the development of plant-based influenza vaccines. One of the first attempts to express influenza antigens in plants was performed by Musiychuk et al. (2007), where transient expression by agroinfiltration of a

Table 7.1 Compilation of the plant-based vaccination models tested for immunogenicity in test animals and clinical trials

Strain	Expression host	Target antigen	Expression strategy	Immunogenic properties	Reference
A/Brisbane/59/07 (H1N1) A/Brisbane/10/07 (H3N2) B/Florida/4/06 A/California/04/09 (H1N1)	*N. benthamiana*	HA	Agroinfiltration	Induced serum anti-HA IgG in test mice, at levels considered protective as estimated by hemagglutination inhibition	Shoji et al. 2012
A/Indonesia/05/2005 (H5N1)	*N. benthamiana*	HA	Agroinfiltration	Showed acceptable safety in a phase I clinical trial. Low humoral immune responses were observed with no dose dependence, suggesting the use of a suboptimal dose	Chichester et al. 2012
A/Indonesia/5/05 (H5N1) A/New Caledonia/20/99 (H1N1)	*N. benthamiana*	HA	Agroinfiltration	Induced protective immunity in test mice against a lethal challenge with a heterologous (A/Vietnam/1194/04) virus	D'Aoust etal. 2008
2009 pandemic H1N1 (pdmH1N1)	*N. benthamiana*	HAC1	Agroinfiltration	Antigenicity was evaluated with sera and cells from individuals immunized with the 2009 pdmH1N1 vaccine. Tobacco-derived HAC1 was recognized by serum antibodies, antibody secreting cells, and induced secretion of IL-2 and IFN-· by Th1-cells	Jul-Larsen et al. 2012
A/Duck/Potsdam/1402_6/1986 (H5N2)	*N. benthamiana*	M2e	Agroinfiltration	Induced protective immunity in test mice against a lethal challenge with influenza virus	Ravin et al. 2012
A/Hatay/2004/(H5N1)	*N. benthamiana*	HA (H5)	Agroinfiltration	Induced potentially neutralizing in test mice	Phan et al. 2013
A/Viet Nam/1194/2004 (H5N1)	*N. benthamiana*	HA(H5)	Agroinfiltration Stable, *Agrobacterium-mediated*	Induced specific antibodies in test mice but not at sufficient levels to inhibit hemagglutination. Vaccine formulation and dosage was estimated as suboptimal	Mortimer et al. 2012
A/Brisbane/59/07 B/Florida/04/06	*N. benthamiana*	HA (H1)	Agroinfiltration	The immunogenic potential in terms of antibody titers induced in mice was similar between the *N. benthamiana*-derived HA than those HA proteins produced in eggs and insect cells	Santiago et al. 2012

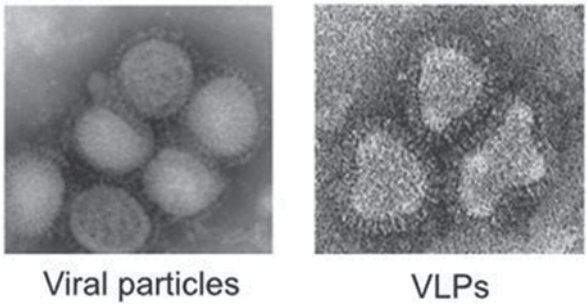

Fig. 7.2 Representative aspect of virus like particles (*VLPs*) under a transmission electron microscopy analysis. Images of viral particles and plant-derived VLPs obtained by the expression of hemagglutinin (*HA*) in *N. benthamiana* plants. (Taken from D'Aoust et al. 2010)

Tobacco mosaic virus (TMV)-based vector (see Chap. 3) was successfully applied to produce a chimeric protein comprising of HA and NA domains of H5N1 influenza virus fused to a thermostable lichenase (LickM). These fusions were able to activate both innate and adaptive immune responses, conferring protection against viral challenge in ferrets (Massa et al. 2007; Mett et al. 2008). Furthermore, Chichester et al. (2012) developed a vaccine candidate based on the recombinant HA protein from the A/Indonesia/05/2005 (H5N1) strain using a transient expression technology in *Nicotiana benthamiana*. This vaccine was evaluated in a phase I clinical trial. Findings derived from this study showed that low humoral response was attained, suggesting a suboptimal dose.

In a report published by Shoji et al. (2012), the production of HAs from four different strains representing the 2008–2009 season was performed using a transient expression system. Antigens were produced at the rate of up to 400–1,300 mg/kg of fresh leaf tissues, with > 70% solubility. Outstandingly, immunization of mice with these HA antigens induced serum anti-HA immunoglobulin (IgG) responses considered protective according to hemagglutination inhibition assays. These developments were also accomplished by research groups at the Fraunhofer Center of Molecular Biotechnology.

Medicago (Canada; www.medicago.com/) is another company that has succeeded on the road of developing plant-made influenza vaccines. This company has also been focusing on the development of vaccines using transient plant-based expression platforms, allowing for a quick development (less than 21 days) of vaccines that exactly matches the specific pandemic strain in circulation (D'Aoust et al. 2010; Fig. 7.2). *N. benthamiana* is being used as expression host, demonstrating that a new vaccine can be produced within 3 weeks from the identification of a new pandemic strain (Landry et al. 2010). Considering the possibility that human strains are capable of mutating and recombining with avian strains (Li et al. 2010; Jackson et al. 2009), Medicago has recently reported positive results from a phase II clinical trial performed to evaluate an avian influenza H5 (AIV) pandemic vaccine candidate, which showed cross-reactivity. This vaccine candidate was found to be safe and well tolerated, and the results were among the most effective obtained by the industry thus far (Medicago Inc. 2013).

In another study, the HA from H5N1 (A/Indonesia/5/05) and H1N1 (A/New Caledonia/20/99) viruses were expressed in agroinfiltrated *N. benthamiana* plants.

HA yields were up to 50 mg/kg biomass and these were assembled correctly into trimers. Moreover, VLPs were assembled and located at apoplastic indentations. The purified H5-derived VLPs were highly immunogenic and immunoprotective: Two doses of 0.5 μg of H5-VLPs conferred complete protection against a lethal challenge with a heterologous virus (A/Vietnam/1194/04; D'Aoust et al. 2008). Based on the promising findings, the next aim comprises the production of a trivalent vaccine formulated with H1N1, H5N1, and B influenza strains. This constitutes the fastest production system for any current pandemic or even seasonal influenza vaccine, reflecting the singular robustness of this approach (Rybicki 2010).

Research groups from institutes and universities have also performed relevant attempts in this field. A Russian group (Ravin et al. 2012) had developed a recombinant vaccine designated M2eHBc, which targets the highly conserved ectodomain of the matrix protein M2 (M2e) fused to the hepatitis B core antigen (HBc). A recombinant viral vector based on potato X virus delivered by agroinfiltration allowed for the expression of the M2eHBc chimeric protein in *N. benthamiana* plants. Expression levels reached up to 12% of the total soluble protein. Interestingly, VLPs exposing the M2e on the surface were detected and then purified to evaluate its immunogenicity in mice. The plant-derived M2eHBc showed a high immunogenicity and, relevantly, a protective effect against a lethal influenza challenge. Therefore, this report augurs a significant potential for this M2e-based candidate influenza vaccine produced in plants under a transient expression system.

Tools for the Design and Production of New Influenza Vaccines

Improvements in the field of vaccination against influenza are envisioned to address the limitation given by the short-term applicability of the current vaccination strategies as well as costs. The development of a universal influenza vaccine is visualized to overcome the genetic variability and the emergent need of being warned against a possible pandemic outbreak. Several academic research groups and biopharmaceutical industries have performed efforts to pursue this goal (Rudolph and Ben-Yedidia 2011). The identification of conserved epitopes has been proposed as the basis for the design of immunogens being able to induce broad immune responses, supporting immunoprotection against several influenza variants. Resources that may aid in this process comprise the epitope maps for influenza proteins, which are currently available at the Influenza Sequence and Epitope Database (ISED 2013). This contains a sequence data collection representing 50,000 influenza A and 5,000 influenza B viruses from 42 countries (Yang et al. 2009; influenza.korea.ac.kr).

A multiepitopic influenza vaccine comprising conserved domains of the circulating strains would be a straightforward strategy to be adopted. Bioinformatics tools can aid in the rational design of a broad range of immunogens. A group in the USA used this useful tool to identify consensus class II HA and NA epitopes derived from strains circulating between 1980 and 2011. These epitopes were selected based on their predicted immunogenicity scores, which were higher or equal with respect to the 95th percentile in these influenza strains (Moise et al. 2013). Therefore, the identification

and sequencing of the influenza strains in conjunction with bioinformatics may lead to the design of universal influenza vaccine candidates. In another approach using immunoproteomics, the direct identification of MHC I-presented epitopes is under way to develop a universal influenza vaccine. Testa et al. (2012) have identified and characterized five epitopes that are conserved among several strains. These conserved T cell epitopes, when combined with the cross-reactive antibody epitope M2e, generate cross-strain-specific, cell-mediated, and humoral immunity and were able to recognize multiple influenza strains. These findings are considered a step toward universal vaccine development and may represent a relevant innovation in the field of plant-based vaccine manufacturing.

Under this context, BiondVax has developed a multiepitopic recombinant vaccine expressed in *Escherichia coli*, composed of 9 conserved domains of HA, NA, and M1, which stimulate both humoral and cell-mediated immunity. Phase I and phase II clinical trials have been conducted with 60 healthy subjects, observing protection against both A and B influenza strains (Atsmon et al. 2012). Another approach accomplished by Dynavax technologies (2013) consisted of the use of the nucleoprotein (NP) and the M2e domain, which were covalently linked in a single immunogen by chemical methods. Promising findings were obtained in terms of safety and immunogenicity in a Phase I clinical trial, which encourage the planification of phase II trial (www.dynavax.com/).

The production of multiepitopic influenza vaccines in plants remains unexplored and is considered a relevant perspective that may avoid the need for generating seasonal vaccines. Taking into consideration the advantages of the plant-based transient expression systems, the idea of a plant-derived universal influenza vaccine might be possible in light of this context.

In terms of protein expression strategies, one relevant approach has consisted of the fusion of elastin-like polypeptides (ELPs). The ELP acts as a tag that is subsequently able to excise itself and rejoin the remaining portions, leading to the formation of aggregates that can be further isolated by centrifugation with a subsequent recovery of soluble monomeric proteins. Using this technology, the protein purification can be accomplished using continuous media flow with high yields, making this process more economical and efficient than conventional expression and purification procedures. This approach is functional even for complex glycosylated proteins produced at high yields and low costs of purification in an easy-handle platform (Fong and Wood 2010; Hassouneh et al. 2012). Interestingly, this technology was applied to avian influenza with promising results (Phan et al. 2013; Phan and Conrad 2011). A transient expression system was used for the production of distinct HA versions from AIV in tobacco plants. Soluble HA trimers were successfully produced and a strong expression enhancement was observed in the ELPylated configuration. An immunization protocol was subsequently performed, observing that the trimeric form of AIV HA enhances the HA-specific immune response compared to the monomeric form. The humoral response induced showed a potent neutralizing activity and reactivity with heterologous inactivated AIV. ELPylation did not influence the functionality and the antigenicity of the stabilized trimers. Applying this modality to human influenza vaccines may open up relevant developments in the field.

Prospective View

Since the first attempt of expressing influenza antigens in plants, reported by the group headed by Yusibov (Musiychuk et al. 2007), a number of candidate vaccines have been reported to date with promising results. Transient expression systems have been mainly used for producing VLPs intended for parenteral administration in pure form.

A special point that deserves attention is the industry investment. According to the present outlook in the industry, influenza plant-based vaccines are envisioned as the first ones to enter into the market due to the adoption of this technology by a couple of companies: Medicago and iBio (ibioinc.com, 2013). The latter applies the technology developed by the Fraunhofer Center of Molecular Biotechnology. Current status reflects large clinical evaluations as the next logical step, particularly completing phase II trial and conducting phase III clinical trials. The use of pure formulations derived from transient expression platforms seems to be a smoother avenue to accomplish the regulatory aspects involved in vaccine approval since these systems are compatible with good manufacturing practices and are administered in a pure form as the conventional vaccines. These influenza vaccines will be probably the first ones facing the issue of social acceptance, which is of critical importance for any technology. Chapter 13 of this book describes how specific actions may allow a proper informative environment to favor acceptance of these new types of vaccines.

A perspective that deserves particular attention consists of exploring the development of oral vaccines using edible crops that may eventually serve as low-cost formulations, since the use of raw plant material avoids complex downstream processing required for the production of parenteral vaccines. However, this aim represents a substantial research activity to obtain a detailed elucidation of the immunology aspects governing the induction of protective humoral responses in the respiratory tract by means of immunization at the gastrointestinal compartment (Pasquevich et al. 2011). For example, some experimental vaccination models have proved the induction of humoral and cellular responses at the respiratory compartment and immunoprotection against respiratory pathogens (Ogra et al. 2001). In a plant-based approach expressing epitopes of *Bordetela pertusis*, Soria-Guerra et al. (2011) immunized BALB/c mice by the oral route and the elicitation of humoral responses at the respiratory tract was observed. Another configuration may comprise the use of plant-based oral vaccines in prime-boost regimens, where oral administration of plant material may enhance the effect of a previous subcutaneous immunization (Alvarez et al. 2006; Arlen et al. 2008). Future studies in these directions may favor the development of new and convenient modalities for influenza immunization.

In conclusion, plant-based technologies have positioned as a viable approach for the production of influenza vaccines, representing one of the best examples of how this technology possesses the potential of modifying the reality of the vaccinology field. The objective of having a new source of low-cost, safe, and efficacious vaccines is expected to be achieved in the near future.

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Chapter 8
Plant-Based Vaccines Against Neglected Tropical Diseases

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Introduction

Tropical diseases (TDs) are defined as those that mainly occur in tropical or subtropical regions. This definition implies that TDs prevail in hot, humid conditions. The neglected tropical diseases (NTDs) represent the most common infections of the world's poorest people, a group sometimes known as "the bottom billion." These tropical infections trap people in poverty through their adverse effects on worker productivity, pregnancy outcomes, and child cognition and development. Recently, the World Health Organization (WHO 2010) developed a list of 17 NTDs (Table 8.1). To be considered under this classification, a disease must meet the following criteria: (1) It prominently affects poor countries; (2) it affects low-income and politically marginalized populations; (3) it does not spread widely as its distribution is restricted by climate and the effects of climate on the distribution of vectors and reservoir hosts; (4) it causes stigma and social discrimination, especially in women; (5) it has a relevant impact on morbidity and mortality; (6) it is relatively neglected by researchers; and (7) it can be controlled, prevented, and possibly eliminated. NTDs impair the lives of 1 billion people worldwide, and threaten the health of millions more. Since 2008, efforts have been made to review and describe the differences in the etiologies, prevalence, and disease burden of the major NTDs according to their regional distribution. In this respect, the prevalence and distribution of the NTDs in the Americas (Sciutto et al. 2000, Schantz et al. 2003), Europe (Torgerson et al. 2011), sub-Saharan Africa, China and East Asia, India and South Asia, Central and Middle East Asia, and North Africa have been previously reviewed according to the report of the WHO/FAO/OIE in 2004.

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Table 8.1 The 17 diseases grouped as NTDs by the WHO (2010). This group of diseases impairs the lives of 1 billion people worldwide

Pathogen	Disease	Causal agent
Parasite	Dracunculiasis	*Dracunculus medinensis*
	Lymphatic filariasis	*Wuchereria bancrofti and Brugia* spp.
	Onchocerciasis	*Onchocerca volvulus*
	Schistosomiasis	*Schistosoma* spp.
	Soil-transmitted Helminthiases	*Ascaris lumbricoides, Trichuris trichiura,* and the hookworms
	Taeniasis/Cysticercosis	*Taenia saginata and T. solium*
	Human echinococcosis	*Echinococcus granulosus and E. multilocularis*
	Fascioliasis	*Fasciola* spp.
	Cutaneous and mucocutaneous leishmaniasis/Visceral leishmaniasis	*Leishmania* sp.
	Chagas disease	*Trypanosoma cruzi*
	Human African trypanosomiasis	*Trypanosoma brucei gambiense*
Virus	Dengue	*Flavivirus*
	Rabies	*Rhabdovirus*
Bacteria	Yaws	*Treponema pertenue*
	Leprosy	*Mycobacterium leprae* and *Mycobacterium lepromatosis*
	Buruli ulcer	*Mycobacterium ulcerans*
	Blinding trachoma	*Chlamydia trachomatis*

The preventive chemotherapy, intensified case management, vector control, water supply, sanitation and hygiene, and veterinary public health can be mentioned among the strategies used to combat NTDs. Epidemiologic data show that it is still necessary to develop accessible new diagnostic tests and medicines to aid in the prevention, cure, and management of complications of NTDs. Moreover, since commissioning infrastructure, appropriate place to allow improved sanitation and hygiene practices, critically depends on economic and political factors, it is often impossible to address this need within a short term in extremely poor communities. In light of this situation, vaccination is considered the best alternative to prevent infectious diseases, as it can lead to a long-term preventive approach. However, since a crucial factor in a successful vaccination program is the logistics costs involved, the development of effective vaccines and low cost are essential in the fight against NTDs. In particular, those vaccines suitable to be orally administered would constitute a realistic and effective alternative to establish successful control programs.

Plant-Based Vaccines: An Alternative for Low-Cost Massive Immunization

Distinct aspects support the oral vaccination as the most attractive route for vaccine administration, especially to fight NDTs: (1) Several pathogens enter the body through mucosal surfaces; thus mucosal immunization could induce local immune

responses that may directly participate as effector immune arm; (2) this approach avoids costly logistical problems, so it is suitable for mass immunization programs; and (3) it is considered the safest administration route as no trained personnel or sterile devices are required.

However, few orally administered vaccines have been commercialized. According to Cripps et al. (2001), this could be due in part to the lack of effective oral delivery systems, which are associated with a delay in the digestive hydrolysis. Studies have showed that these limitations could be overridden using plant cells for oral vaccine delivery. Plant systems enable us to produce antigens at low costs, avoiding the costly step of purification of antigens and expensive technologies for artificial antigen encapsulation. Therefore, they are easily accessible and can be an attractive approach for mass immunization in poor countries (Daniell 2006).

Plant-based orally administered vaccines are formulated with biomass from transgenic plant cells or tissues expressing antigenic immunoprotective heterologous proteins. Plant biomass can protect antigens from further degradation in the digestive tract, enabling them to reach the gut-associated lymphoid tissue (GALT). This approach has proved to be an effective and accessible strategy for immunization. The companies currently developing this type of vaccines and the number of products that are close to be marketed constitute a good evidence of the viability and current advances in this technology (Yusibov et al. 2011). Currently, Protalix BioTherapeutics has on the market a plant-derived product named ELELYSO, a recombinant hydrolytic lysosomal glucocerebroside-specific enzyme indicated for long-term enzyme replacement therapy (ERT) for adults with confirmed diagnosis of Type 1 Gaucher's disease. This case exemplifies the high potential of plant-derived biopharmaceuticals to be marketed in the near future.

As NTDs are characterized by having a negative impact on poor populations, it would be expected that they should be a priority in the plant-based vaccine development field. Plant-based vaccine developments are under way for the following NTDs: soil-transmitted taeniasis/cysticercosis, human echinococcosis, several helminthiases, dengue, and rabies. This chapter describes the research efforts carried out by various research groups around the world to develop plant-based vaccines against these NTDs, which reflect a potential for conducting field evaluations and also a few formulations in the market in the near future.

Cysticercosis

Neurocysticercosis (NC) is caused by the *Taenia solium* metacestode when located in the human brain. The great majority of NC cases occur in developing countries (Rodriguez-Canul et al. 1999), but it is being considered as an emerging disease in the developed world due to human immigration (Kraft 2007). NC is one of the most frequent parasitic diseases of the central nervous system (CNS) and the main cause of secondary epilepsy worldwide (Dumas and Preux 2008). Cysticercosis outside the CNS (XNC) is frequently reported in Asia and Africa, but less so in America (Kumar et al. 1996). More than 80 % of the world's 50 million people who

are affected by epilepsy live in endemic developing countries. Porcine cysticercosis mainly affects the health and livelihoods of subsistence farmers in developing countries of Africa, Asia, and Latin America, as it reduces the market value of pigs because of decommission (Torgerson et al. 2011). Currently, no exact data on the world prevalence of cysticercosis are available. WHO estimated in 2004 that about 2.5 million persons are living with intestinal taeniasis globally. NC is an endemic disease linked to poverty and ignorance rooted by complex economic and social networks that underlie cestode transmission. Its eradication remains a major challenge, particularly in non-developed countries where cestode life cycles are firmly established in the social, cultural, and economic contexts.

However, cysticercosis is a preventable disease and has been declared eradicable by the International Task Force for Disease Eradication since 2003, even though no sustained national programs are ongoing in any of the endemic countries. Accordingly, its prevalence remains stable in different health institutions of endemic countries like Mexico (Fleury et al. 2010, 2012; Canseco-Avila et al. 2010), and is growing in non-endemic countries due to immigration (Serpa and White 2012).

Several tools to effectively interrupt transmission have been developed and field evaluated, i.e., health education, detection, and treatment of tapeworm carriers, and pig vaccination (Sarti et al. 1997, 2000; Pawlowski et al. 2005; Huerta et al. 2001; Morales et al. 2008; Assana et al. 2010). Between them, increasing porcine resistance to the parasite establishment by improving the specific host immune status through vaccination is a realistic strategy to impact transmission that does not require the development level of the marginalized populations worldwide. The interruption of transmission by pig vaccination is of special interest, considering the difficulties of developing human vaccines. Indeed, the essential role of pigs as obligate intermediate hosts in the parasite life allows us to effectively interrupt the transmission by pig vaccination. This is a feasible strategy accepted by pig owners, since it is not confiscatory. Vaccination is also economy conscious, as the pigs will resist infection even though rustically bred, and the meat will reach higher prices if it meets inspection standards in abattoirs.

Several vaccines have been developed and field-trial tested. All of them are effective but injectable vaccines (Huerta et al. 2001; Morales et al. 2008; Assana et al. 2010). Their parenteral application implies a costly and logistic tool and limits its use in nationwide programs. Moreover, injectable vaccines require a risky capture of wild roaming pigs by two or three trained persons.

An orally administered vaccine would elude these difficulties, since it could be delivered by the pigs' owners when they are fed. In addition, an oral vaccine is particularly attractive for the prevention of orally acquired infections caused by organisms like *T. solium*.

S3PVac: A Papaya-Based Vaccine

An injectable vaccine against pig cysticercosis, named S3Pvac, based on three peptides (KETc7, KETc1, KETc12), expressed by another cestode (*T. crassiceps*),

was developed. S3Pvac, when synthetically and recombinantly expressed, reduced infection in about 50% of the number of infected pigs and in 80–90% of the number of established cysticerci under natural conditions of transmission (Huerta et al. 2001; Morales et al. 2008). In an effort to develop an oral version of the vaccine, KETc1, KETc12, and KETc7 were expressed in three independent papaya embryogenic cell lines, obtained by biobalistic (Hernández et al. 2007). The vaccine, composed of the three clones, was designated as S3Pvac-papaya. The expression of the respective peptide in each clone was confirmed at the transcriptional level by reverse transcription polymerase chain reaction (RT-PCR). Soluble extracts from the transgenic papaya clones were found to be immunogenic when subcutaneously administered to mice. Indeed, the three clones expressing the vaccine peptides induced a high level of protection against murine cysticercosis. These achievements highlight the great potential of this technology to render a highly effective and affordable vaccine against cysticercosis.

Recently, S3Pvac-papaya also demonstrated a high protective capacity against *T. pisiformis* cysticercosis (Betancourt et al. 2012) when orally administered to rabbits. Furthermore, oral S3Pvac-papaya induced a protection level as high as the injectable synthetic version of the vaccine, and even higher than the recombinant S3Pvac version expressed in filamentous phages. S3Pvac-papaya also demonstrated to be immunogenic (Fig. 8.1) in pigs when orally administered (Hernández et al. 2007). Currently, this vaccine is being evaluated in pigs under oral immunization schemes (Edda Sciutto, personal communication).

To further explore the potential of an oral multi-epitope vaccine, the addition of antigens that have shown to elicit protective immunity was considered. In this respect, the most promising candidate is TSOL18/HP6. This antigen has been proved capable of protecting pigs against *T. solium* experimental infection (Flisser et al. 2004). More recently, its protective effect was evaluated when coadministered with the anthelminthic oxfendazole on a field-trial study in rural communities in Cameroon with amazing results (Assana et al. 2010). Later on, it was further evaluated on the field (Jayashi et al. 2012) combined with another protective recombinant antigen, TSOL16 (Gauci et al. 2012). Although the protective capacity of these antigens when orally administered has not been evaluated, it is feasible that if expressed in an adequate delivery system, they could be immunogenic and could be considered to be included as additional epitopes in the plant-derived S3Pvac vaccine.

Human Hydatidosis

Hydatid disease, caused by *Echinococcus granulosus* or *E. multilocularis,* is another important cestodiasis that affects human and veterinary health (Moro et al. 2009). It is also considered as an NTDs. Cystic and alveolar echinococcosis are caused in humans due to the ingestion of eggs of *E. granulosus* or *E. multilocularis,* respectively, shed in the feces of dogs that harbor the adult stages of these tapeworms. Echinococcosis has a global distribution and causes serious morbidity and even death if left untreated. Different vaccination candidates against hydatidosis have been reported.

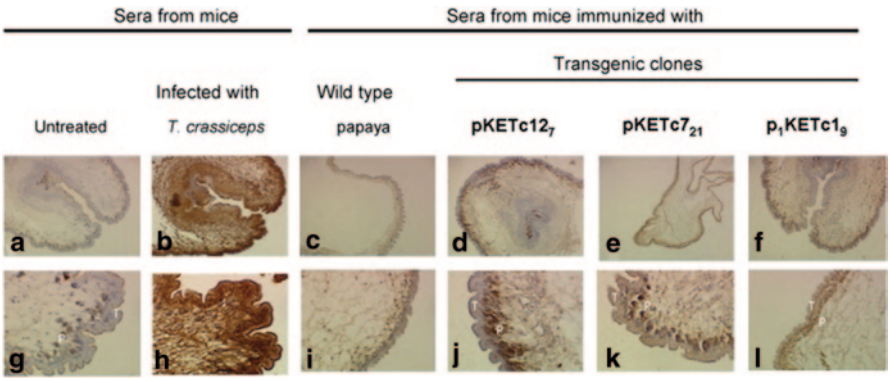

Fig. 8.1 Immunohistochemistry to detect specific antibodies in sera from mice immunized with the S3Pvac papaya-based vaccine. Sections of *Taenia crassiceps* tissues were processed and incubated with sera from untreated mice (**a**, **g**), from *T. crassiceps*-infected mice (**b**, **h**), and from mice immunized with ETgpC (**b**–**f** and **h**–**l**). KETc7 was detected in tegument (*T*) and parenchyma (*P*), while KETc1.6His was only detected in tegument and KETc12.6His in parenchyma. (Taken from Hernández et al. 2007)

The antigen candidate most thoroughly evaluated against hydatidosis is named Eg95 (Lightowlers et al. 1996, 2004). This injectable recombinant vaccine was proved to be efficient in an extensive field trial performed in sheep, goats, and cattle (Zhang et al. 2008, 2009). Eg95 has been registered for use in China and Argentina, and it has been produced commercially for large-scale use in control programs, assuming that its high cost will be heavily subsidized by health authorities. However, this is a costly vaccine, because it requires being administered in two doses, with an additional booster to maintain the vaccine immunity. Moreover, the parenteral administration is not suitable for large-scale application (Barnes et al. 2012). The use of an appropriate delivery system to improve Eg95 immunity and reduce production costs is under development (Cross et al. 2011). Other approaches are also under experimentation. Among them figure EgTrp-tropomyosin and EgA31-tropomyosin, antigens expressed in the larval and the adult stages of the parasite, which elicit promising protective responses in experimental trials (Barnes et al. 2012). While these approaches seem feasible, the use of plant cells as delivery vectors for an orally administered vaccine would yield convenient vaccines as well, at lower costs.

Approaches of Plant-Based Hydatidosis Vaccines

Several plant-based vaccine candidates against hydatidosis have been reported. Both EgA31 by itself and combined with Eg95 were used to produce transgenic alfalfa plants by nuclear transformation. The immunogenic properties of these cells were evaluated in the hydatidosis mouse model. Oral and intranasal immunization with transgenic alfalfa significantly reduced larval size and elicited a pro-inflammatory response, probably involved in this protective capacity (Ye et al. 2010a, b)

It is also of interest to mention that the S3Pvac vaccine can protect not only against cysticercosis but also against hydatidosis, as shown in a field-trial test performed on 187 vaccinated and 204 control pigs. Macroscopic and histological evidences showed that protective efficacies of S3Pvac-phage vaccination against porcine cysticercosis and hydatidosis are of 61.7% and 56.1%, respectively (Morales et al. 2011). Then, S3Pvac expressed in papaya, injectable or orally administered, may also possibly prevent both infections.

Helminthiasis

Soil-transmitted helminths cause most of the infections affecting the poorest populations. As these pathogens enter the human body through mucosal surfaces, oral immunization could be a promising strategy to prevent such infections. The main causal agents of these infections are *Ascaris lumbricoides, Trichuris trichiura, Ancylostoma duodenale,* and *Necator americanus.* According to recent estimates, *A. lumbricoides* infects over 1 billion people, *T. trichiura* 795 million, and hookworms 740 million. The great majority of soil-transmitted helminth infections occur in sub-Saharan Africa, the Americas, China, and East Asia. Infection is caused by ingestion of eggs from contaminated soil (*A. lumbricoides* and *T. trichiura*) or by active skin penetration by larvae present in the soil (*A. duodenale* and *N. americanus*). Soil-transmitted helminths produce a wide range of symptoms, including intestinal manifestations (diarrhea and abdominal pain), general malaise, and weakness, which may affect working and learning ability and impair physical growth. In particular, hookworms cause chronic intestinal blood loss leading to anemia (Lustigman et al. 2012).

Currently, anthelminthic drugs are being globally distributed in an effort to control these intestinal parasitoses, with the potential probability to develop drug resistance (Epe and Kaminsky 2013). In spite of these efforts, intestinal parasitoses remain highly prevalent worldwide. The high growing childhood population, particularly in endemic countries, the poverty conditions in which they live, and the lack of immunity induced by the infection, which demands periodic administration of these drugs, promote their maintenance (WHO 2005). In spite of the lack of effective immunity induced by a primary infection against reinfections, some protective antigens have been reported (Chen et al. 2012). Regardless of all the international efforts and the relevance to develop an effective vaccine against these parasitic infections, worm control remains a major challenge.

Approaches of Plant-Based Anti-Helminthiasis Vaccines

Very few studies on plant cells expressing helminthic antigens have been performed. Only a couple of reports on *A. suum* antigen production have been published to prevent the porcine infection (Tsuji et al. 2003, 2004). The extensive similarities

in natural history, pathology, and antigenic composition between *A. suum* and *A. lumbricoides* allow us to consider that protective vaccine antigens against one species could be useful against the other (Matsumoto et al. 2009).

A. suum infection begins when embryonated eggs encapsulating third-stage larvae (L3) are ingested by host animals. Parasites hatch in the host's small intestine, migrate to the liver and lungs via the portal vein, and finally reach the cecum and/or proximal colon, where they develop into adult worms. *A. suum* can also infect humans, a finding that points to its relevance as a zoonotic parasite (Maruyama et al. 1996; Nakamura-Uchiyama et al. 2006). Previous studies showed that animals can be protected from *A. suum* infection by immunization with L3 or with a cuticle component (Hill et al. 1994). This evidence led to describe a protective 16 kDa antigen (As16), which is expressed in the intestine, hypodermis, and cuticles of larva and adult stages of *A. suum* (Tsuji et al. 2003). It has been proved that antibodies elicited by immunization with As16 are able to kill *A. suum* L3 (Tsuji et al. 2004), a finding that supports the possibility to use this protein as a viable vaccine antigen against this parasite.

In 2009, Matsumoto et al. reported the evaluation of rice plants for As16 production as a chimeric protein fused to the cholera toxin B subunit (CTB). Expression levels up to 50 μg/g seed (Matsumoto et al. 2009) were observed. Feeding mice with the transgenic rice seeds elicited an As16-specific serum antibody response when administered together with CT as a mucosal adjuvant. Even though no response was observed when no adjuvant was used, subcutaneous booster immunization, with bacteria-expressed As16, successfully induced antibody responses; therefore, a priming effect by the transgenic rice is postulated. Notably, mice orally immunized with transgenic rice/CT showed lower lungworm burdens after a challenge with *A. suum* eggs (Fig. 8.2). This suggests a good potential of the rice-derived antigens as a low-cost vaccine candidate to control *A. suum* infection in animals, but it could also serve as a model for developing human vaccines against other helminthiasis. Cross-protective immunity against *A. lumbricoides,* for example, should be explored as it could lead to another successful application for this approach. The same research group expressed the 14 kDa protective surface antigen from *A. suum* L3 in rice, which was fused to the mucosal carrier CTB; but in this case, the expression was driven by the endosperm-specific *glutelin*-B promoter. This strategy allowed for the production of the expected recombinant proteins at levels up to 1.5 μg per seed. This study represents an advance in the field that justifies further functional studies (Nozoye et al. 2009). Further efforts in this field require assessing the immunogenic properties of these transgenic plants.

The dramatic rise in drug-resistant helminths of veterinary importance have raised concerns over the long-term use of current drugs (Epe and Kaminsky 2013). Drug-discovery programs, based on the screening of chemical compounds against parasitic or non-parasitic helminths, will require to be expanded. The study of immune-mediated expulsion of adult worms could potentially benefit from this process through the identification of novel anthelminthic molecules. A novel immunotherapy approach is the administration of interleukins 4 and 13 instead interleukin-4 (IL-4)/IL-13 to promote adult worm expulsion. In particular, an increase in the intestine of the resistin-like

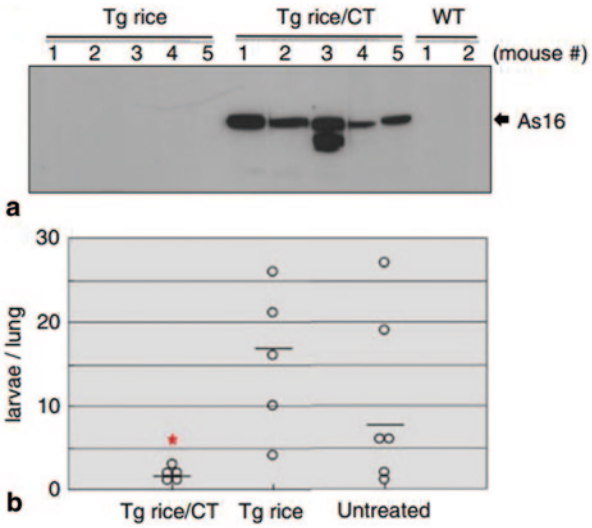

Fig. 8.2 Oral immunogenicity and protective effect of the rice-derived As16 vaccine. **a** Induction of As16-specific IgG in serum by administration of Tg rice seed. BALB/c mice were fed Tg rice with or without cholera toxin (CT) as adjuvant. Sera from mice immunized with Tg plus CT show positive reactivity against bacterially expressed As16 protein. **b** Parasite load in the lungs of immunized mice. Mice were orally infected with *Ascaris suum* eggs 1 week after the last immunization, and the L3 that migrated to the lungs were counted. (Taken from Matsumoto et al. 2009)

molecule beta (RELMb) levels results in an inhospitable environment for the worm and interferes with essential worm functions such as feeding (Herbert et al. 2009), a mechanism independent of the classical immunological pathways involved in worm expulsion (Th2 or M2 macrophages activation). As a number of cytokines retaining their functional properties have been produced in plants, the use of this platform to accomplish cytokine-mediated treatment of helminthiasis is also a possibility (Gutierrez-Ortega et al. 2004; Magnuson et al. 1998).

Rabies

Rabies is a viral zoonotic infection of the CNS caused by a lyssavirus. Rabies virus causes the highest number of deaths among all human pathogenic viruses. In 2005, WHO pointed out that 50% of the yearly reported victims were 15 years old or younger. Of these cases, 95% occur in Asia and Africa, and 99% of them are transmitted by dogs. In general, this transmission occurs in exposed humans through a bite by a rabies virus-infected animal or through mucosal contact with virus-contaminated fluids. Therefore, rabies is considered one of the most NTDs in developing countries, with the greatest burden put on poor rural communities and disproportionately on children. The efforts by WHO to reduce rabies burden and to

eradicate the disease in humans involve coordinated efforts to procure and deliver safe and efficacious rabies vaccines in those countries where they are most needed, to achieve preventive immunization in animals and preexposure and postexposure prophylaxis in humans (WHO 2005).

Rabies virus has a single-stranded RNA genome encoding for five structural proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and RNA-dependent RNA polymerase (L). Among these proteins, G protein is the most important in viral pathogenesis, which may function as a protective antigen, since it is the only target for neutralizing antibodies (NAs) that provide full protection against virus challenge. Several research groups have produced this glycoprotein in recombinant yeast or insect cells or in transgenic plants. The immunogenicity of the recombinant protein has been found to be different according to the expression system employed, probably due to the structural complexity of the rabies virus glycoprotein, which carries two N-linked oligosaccharide branches (Shakin-Eshleman et al. 1992). Most rabies virus NAs bind to conformation-dependent epitopes on the native glycoprotein that seem to be expressed preferentially as multimeric proteins rather than as the monomeric forms of cleaved glycoprotein, secreted by infected cells or commonly produced in recombinant expression systems (Ertl et al. 2009).

Although rabies is a vaccine-preventable disease, effective prevention in humans with category III bites requires the combined administration of rabies immunoglobulin (RIG) and rabies vaccine. Cell culture-based rabies vaccines have become widely available in developing countries, virtually replacing the inferior and unsafe nerve tissue-based vaccines. Limitations inherent to the conventional RIG of either equine or human origin have prompted scientists to look for monoclonal antibody-based human RIG as an alternative. This approach is attractive but has a severe drawback, as the production of such vaccines is costly, making them less accessible to the general population. Different alternative approaches expressing potential antigenic proteins have been developed for reliable and accessible vaccines. Among them, plant-based vaccines are the most promising candidates.

Approaches of Plant-Based Rabies Vaccines

Several approaches of plant-based vaccines against rabies have been reported. In a pioneering study, tomato plants were engineered at the nuclear level to express the G-protein, which was successfully immunoprecipitated and detected by Western blot from leaves and fruit. Electron microscopy of leaf tissue using immunogold-labeling and antisera specific for rabies G-protein showed that it was located in Golgi bodies, vesicles, plasmalemma, and cell walls of vascular parenchyma cells, suggesting that tomato can serve as a functional expression platform for this vaccine (McGarvey et al. 1995). Later, Yusibov et al. reported the expression of the antigenic protein designated CPDrg24, which is comprised of the G5-24 B cell epitope from rabies glycoprotein and a 31D T cell epitope from rabies nucleoprotein, fused with *Alfalfa mosaic virus* (AIMV) coat protein (CP). This chimeric protein was

cloned into the 30BRz vector, which allowed for its expression in infected plants, yielding viral particles that were successfully purified from infected plant tissue and used to immunize mice intraperitoneally. High serum titers of rabies-specific antibodies were detected by enzyme-linked immunosorbent assay (ELISA) 14 days after the last CPDrg24 immunization in mice immunized either with or without complete Freund's adjuvant. Thus, plant-produced rabies virus antigen is capable of inducing an immune response in mice in an adjuvant-free system. Moreover, in an in vitro assay, the authors proved that these antibodies are capable of neutralizing CVS-11 strain rabies virus (Yusibov et al. 1997).

Subsequently, the expression of chimeric Drg24 rabies virus peptide in virus-infected spinach leaves was reported, showing that either intraperitoneally or orally immunized mice developed local and systemic immune responses. Both groups were subjected to challenge experiments, and 40% of the intraperitoneally-immunized animals were protected against lethal challenge. On the other hand, oral administration of the antigen not only stimulated serum IgG and IgA syntheses but also ameliorated the clinical signs caused by intranasal infection with an attenuated rabies virus strain (Modelska et al. 1998).

In another approach, Yusibov et al. (2002) reported the production of a chimeric peptide carrying antigenic determinants of the G-protein (amino acids 253–275) and nucleoprotein N (amino acids 404-418), fused to the AIMV CP. This protein was expressed in transgenic tobacco (*Nicotiana tabacum* L. cv. Samsun-NN) plants, providing transreplicative functions for full-length infectious RNA3 from A1MV (NF1-g24). In addition, *N. benthamiana* and spinach (*Spinacia oleracea*) plants were used as expression systems using an autonomously replicating *Tobacco mosaic virus* (TMV) lacking the native CP (Av/A4-g24). The recombinant virus was purified from *N. tabacum* cv. Samsun NN cells and shown to keep its capacity to elicit humoral responses in mice when parenterally administered. Moreover, recombinant virus-containing unprocessed raw spinach leaves conferred protection to mice against oral challenge. Based on these results, this research group assessed its efficacy in a pilot study on human volunteers. Of the five volunteers previously immunized with the conventional vaccine, three specifically responded against the peptide after ingesting spinach leaves transfected with the recombinant virus, while five of nine non-immune individuals (fed with the same material) exhibited significant antibody responses to either rabies virus or AIMV. After a single dose of conventional rabies virus vaccine, three unvaccinated individuals showed detectable levels of NAs against rabies virus (Fig. 8.3). These findings show the potential for the plant virus-based expression systems as supplementary oral booster in rabies immunization schemes (Yusibov et al. 2002).

Some research groups have attempted to engineer virus CPs to use them as carriers for genetically fused rabies-specific antigen. Such carrier proteins could have the potential to self-assemble and form recombinant virus particles, which are often highly immunogenic and display the desired epitopes on their surfaces. Indeed, high G-protein expression levels were obtained through optimized codon usage system that also included the native signal peptide of the pathogenesis-related protein PR-S, as well as the endoplasmic reticulum retention signal (Ashraf et al.

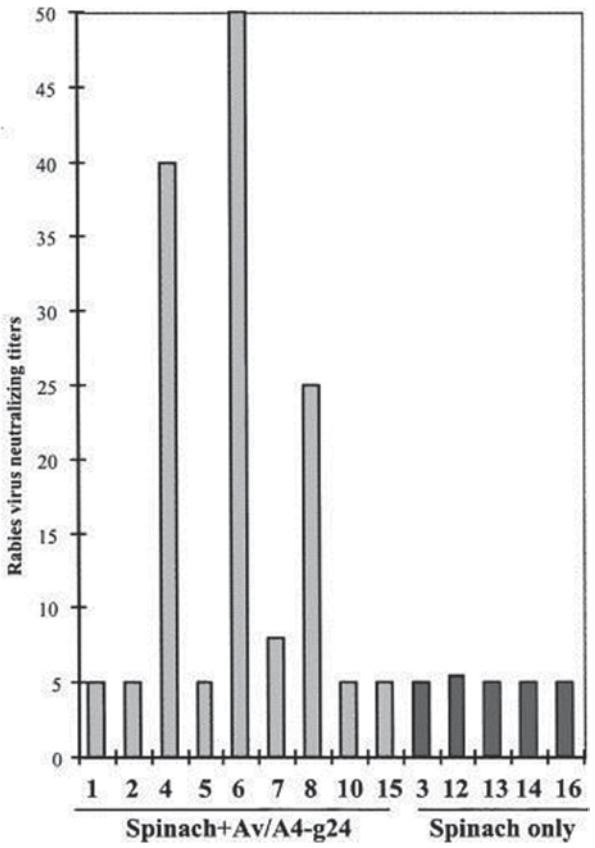

Fig. 8.3 Rabies virus-neutralizing antibody titers in sera of individuals who received a single dose of commercial rabies vaccine and a boost with the spinach-derived vaccine. Numbers on the axis indicate volunteer designations. (Taken from Yusibov et al. (2002)

2005). Tobacco plants transformed with this construct at the nuclear level were able to express the protein at levels up to 0.38% of the total soluble protein (%TSP) According to the study, mice intraperitoneally-immunized with the plant-derived G-protein purified from tobacco leaf microsomal fraction showed significant immune responses as high as those induced by the commercial inactivated virus vaccine. More importantly, this immunization scheme induced complete protection against a lethal intracerebral rabies virus challenge. This approach constituted a significant step towards the development of a feasible and accessible vaccine against rabies (Ashraf et al. 2005).

Rabies surface glycoprotein (G-protein) has also been expressed in tobacco plants as a fused protein with the B subunit of CT, in an effort to use this protein as an immunogenic carrier. The expected recombinant protein was accumulated at levels up to 0.4% of TSP leaves and it was functionally active in the GM1 binding assay, having a higher affinity for GM1 than for the native bacterial CTB; these results have interesting implications since this binding activity is associated with a higher immunogenic potential. The pentameric fusion was immunoreactive both with anti-CT and anti-rabies antibodies, suggesting that the antigenic determinants of both components were preserved. Nonetheless, the immunoprotective ability

against rabies is still a pending objective (Roy et al. 2010). Immunogenicity evaluation for these proteins and their capacity to neutralize the virus in humans also remain a high-priority perspective.

On the other hand, transgenic maize expressing the Vnukovo strain rabies virus glycoprotein (G) has been developed by Loza-Rubio et al., (2008). Interestingly, the expression levels reached up to 1 % of TSP. This research group evaluated the immunogenicity of the heterologous protein when orally administered. Adult mice received a single oral dose of kernels containing 50 μg of G-protein and 90 days post vaccination, they were challenged with a lethal dose of a vampire bat rabies virus. Notably, a 100 % protection was recorded in this experiment. The G-protein from Vnukovo strain provides rabies cross protection; thus, these corn lines are promising candidates for the formulation of a highly effective rabies vaccine (Loza-Rubio et al. 2008).

All these attempts to express virus antigenic proteins in different plant systems point to its future use in applied vaccinations as alternatives for both animals and humans.

Dengue

Dengue is caused by a virus of the family *Flaviviridae* (DENV). Four dengue virus serotypes, designated DENV-1–4, have been found concomitantly in different world regions. This virus is related to the yellow fever virus (YFV), hepatitis C virus (HCV), and the West Nile (WNV), Japanese (JEV), and St. Louis encephalitis viruses. Each virion contains a single positive-strand RNA coding for dengue viral proteins in a long open reading frame comprising capsid (C), premembrane (prM), envelope (E), and non-structural (NS) 1–5 genes. Human infection with DENV results in either an asymptomatic or a symptomatic disease, ranging from classical dengue fever (DF) to more severe cases of dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS; Konishi et al. 2000).

Dengue is an acute febrile, mosquito-borne viral disease. In recent years, transmission rate has increased predominantly in urban and semi-urban areas in tropical and subtropical regions. The incidence of this infection has grown dramatically around the world in recent decades. Over 2.2 million cases were reported in 2010 in the Americas, South-East Asia, and the Western Pacific (WHO 2012). At present, no vaccines against dengue are available. However, various strategies to develop dengue vaccine candidates, including models of attenuated, recombinant, subunit, chimeric, and DNA vaccines, have been attempted (Saejung et al. 2007).

The viral proteins prM, E, and NS1 are considered relevant to provide immunity, since passively transferred antibodies against each of these proteins have been reported to protect mice from lethal challenge (Lai et al. 2007). Therefore, prM, E, and NS1 genes have been used to develop dengue subunit vaccines (Konishi et al. 2000). Most of these strategies have been focused on the dengue virus E protein. This 495-aa protein consists of three structurally distinct domains, named I, II, and

III (Modis et al. 2003). This component constitutes the major structural protein exposed on the mature virion surface, having a main role in host cell attachment and viral entry. Thus, it has been considered as the primary antigen to attain protective immunity (Saejung et al. 2007).

Despite significant efforts exerted in many countries, no commercially viable dengue vaccine is available. Currently, attention is focused on developing either live attenuated vaccines or live attenuated chimeric vaccines using a variety of backbones. Alternative vaccine approaches, such as whole inactivated virus and subunit vaccines, are in early development stages, but each poses different drawbacks. Subunit vaccines offer the advantage of providing a well-defined antigen, without adding the risk of introducing foreign genetic material into the immunized subject. Preliminary trials of subunit vaccines (using dengue E protein) in rhesus monkeys have shown promising results. However, the primary disadvantages of dengue subunit vaccines are the low expression levels of dengue proteins in mammalian or insect cells, as well as the inclusion of potential antigen contaminants from the delivering mammalian or insect cells. To circumvent, in part, these disadvantages, plants have been considered to be effective alternative production systems for subunit vaccines, as at least high levels of expression can be achieved (Malabadi et al. 2011).

Approaches of Plant-Based Dengue Vaccines

In a pioneering approach, domain III of dengue 2-envelope protein (D2EIII, 298–400-aa) was successfully expressed in *N. benthamiana* cells using a TMV-based transient expression system. The recombinant protein was immunoreactive to both anti-D2EIII polyclonal and anti-His tag antibodies. Mice intramuscularly immunized with the plant-derived vaccine elicited anti-dengue virus humoral responses. Moreover, neutralizing activity against type-2 dengue virus by sera from immunized animals was high, a result that clearly indicated that the TMV expression system produces a dengue virus-derived antigen in plant cells that exhibits appropriate antigenicity and immunogenicity (Saejung et al. 2007). The observed response, however, was induced only when an adjuvant was coadministered with the antigen. Therefore, planning alternative strategies to achieve a highly immunogenic formulation is a mandatory step, for example, adjuvant co-expression or an antigen-adjuvant genetic fusion that allows the induction of higher immune responses even by oral immunization. It is well known that the CTB subunit and the heat-labile *E. coli* enterotoxin B subunit can serve as advantageous carrier proteins with adjuvant properties, and in addition, they have been expressed in a number of plant species, in which they retain their antigenic and immunogenic properties.

This approach has been explored by other research groups. CTB was used as a carrier for the poorly immunogenic EIII domain (297–394-aa). The protein, called CTB-EIII, was produced in tobacco plants following the standard *Agrobacterium*-mediated transformation procedure. This system allowed for producing the CTB-EIII protein at levels close to 0.019% of TSP. Interestingly, this plant-derived

antigen retained the ability to bind GM1, which is critical for its biological activity. Immunogenicity in mice would be the next logical step in this project. It is also of interest to express this protein at the chloroplast level, in search for better yields (Kim et al. 2010).

In another adjuvant-based approach, an E-protein truncated version was expressed in *N. benthamiana* using deconstructed viral modules as delivery system (Martínez et al. 2010). The following configurations were tested: (1) a truncated version of E (Et), lacking the membrane anchor domain; (2) the co-expression of Et with DV structural proteins C and prM (CMEt); and (3) the fusion of HBcore with DV serotype 2 domain III of the envelope protein (DV2d3) (HBcore-DV2d3). All these constructions proved to be antigenic, since they reacted with the corresponding antibodies (anti-E and anti-HBcAg). Therefore, future efforts in this field should comprise immunogenicity studies.

A transplastomic approach was recently assessed through the expression of the dengue-3 serotype polyprotein (prM/E) consisting of partial C, complete prM, and truncated envelope (E) proteins (Kanagaraj et al. 2011). This system was successfully attained in lettuce chloroplasts. The rationale of this approach is to produce an antigen capable of assembling into virus-like particles (VLPs), and therefore assuming a highly immunogenic form. Using western blot analysis, the authors proved that prM/E polyprotein was expressed in different forms: as monomers (≈65 kDa) or possibly heterodimers (≈130 kDa), or multimers. VLPs of ≈20 nm diameter in chloroplast extracts from transplastomic prM/E protein-expressing lettuce cells were detected, although their immunogenicity still requires to be analyzed. A comparison of the immune responses attained by this antigen with monomeric approaches would be interesting to ascertain whether or not multimeric forms possess enhanced immunogenic properties.

Overall, this panorama shows that plant-based vaccine candidates have the potential of eliciting specific immune responses against dengue. However, it is necessary to evaluate whether the elicited antibodies are capable of preventing the virus from entering the host cell. An effort to obtain an effective plant-based vaccine may also be directed to achieve the simultaneous expression of prM, E, and NS1 proteins with the goal of inducing a broad specific immune response in a single formulation.

Prospective View

It is notable that various research groups over the world are currently making efforts to develop vaccines against NTDs (see summary in Fig. 8.4). However, many challenges remain to be addressed. It is expected that in the following years the available models can yield enough data to encourage clinical trials for these vaccines. In this situation, plant-based vaccines are likely to have a profound impact on NTD prevention. There is, however, a significant number of NTDs not considered yet for plant-based vaccine prevention. This demonstrates that the development of plant-based vaccines against NTDs is still limited, and shows the necessity to expand

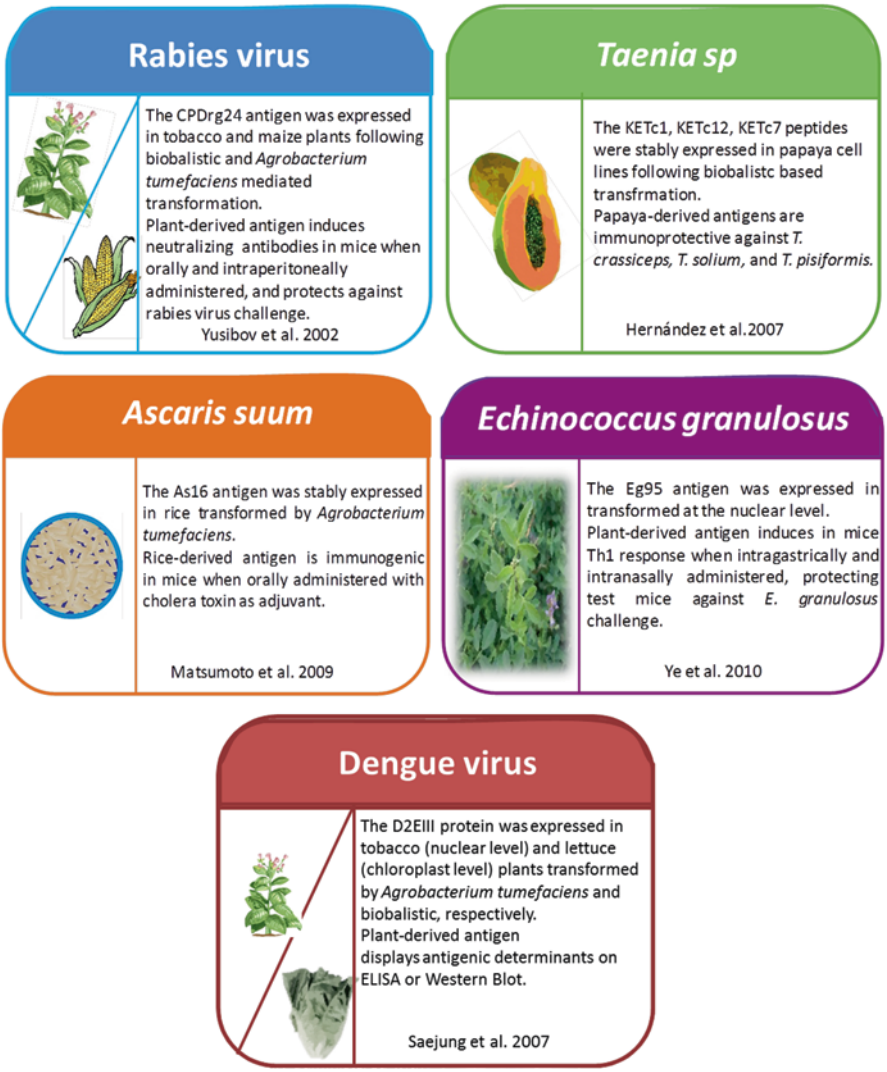

Fig. 8.4 Schematic summary of advanced developments addressing plant-based vaccination against specific neglected tropical diseases. s.c., subcutaneous; i.m., intramuscular; i.p., intraperitoneal; i.g., intragastrical; i.n., intranasal; p.o., oral; ELISA, enzyme-linked immunosorbent assay

the application of this biotechnological tool to other immunoprotective antigens to assess new experimental models and yield data supporting the approach in other cases of interest. Since only a modest attention has been paid to NTDs in the field of plant-based vaccination, there is a clear necessity of addressing projects where this biotechnological approach can be applied as low-cost vaccine models. Although several edible crops can be efficiently transformed, few groups have reported the use of edible crops to assess plant-based vaccination models. This aspect is also

of special importance, since it will constitute a step forward in the development of products suitable for evaluation in clinical trials. The discovery of new immunogens, along with an increased knowledge on the immunological mechanisms mediating immunoprotection, may lead in the near future to new developments that will facilitate the exploitation of plants as biofactories and delivery systems for low-cost vaccines, with the potential to improve the life quality of people affected by NTDs.

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Chapter 9
Plant-Based Vaccines Against the *Human Immunodeficiency Virus*

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Introduction

The *human immunodeficiency virus* (HIV) is the causative agent of acquired immune deficiency syndrome (AIDS), which is a worldwide health problem. Approximately 34 million HIV-positive individuals are reported to be infected with HIV according to UNAIDS. HIV is an enveloped RNA virus responsible for slow and progressive infection that impairs immune function mainly by trough depletion of CD4 lymphocytes (Karlsson Hedestam et al. 2008).

The development of the highly active antiretroviral treatment (HAART) in industrialized countries has resulted in some progress as it reduces disease progression, thus converting it to an effectively manageable chronic disease (Palella et al. 1998; Walensky et al. 2006). However, cost, secondary effects, and emergence of multiclass drug-resistant variants present limitations that render the development of alternative therapeutic and/or prophylactic treatments necessary (Mallon 2007; Richman et al. 2009; Gottlieb et al. 2009). Within this context, vaccination is deemed the most desirable and beneficial approach to fighting the HIV/AIDS epidemic.

Serious efforts have been undertaken to develop an effective vaccine against HIV; these included the use of an inactivated virus, protein subunits, synthetic peptides, DNA vaccines, and viral vectors (Gamble and Matthews 2010). Emphasis on designing subunit vaccines capable of eliciting broad humoral and cellular responses has received much attention as they should ideally protect against HIV

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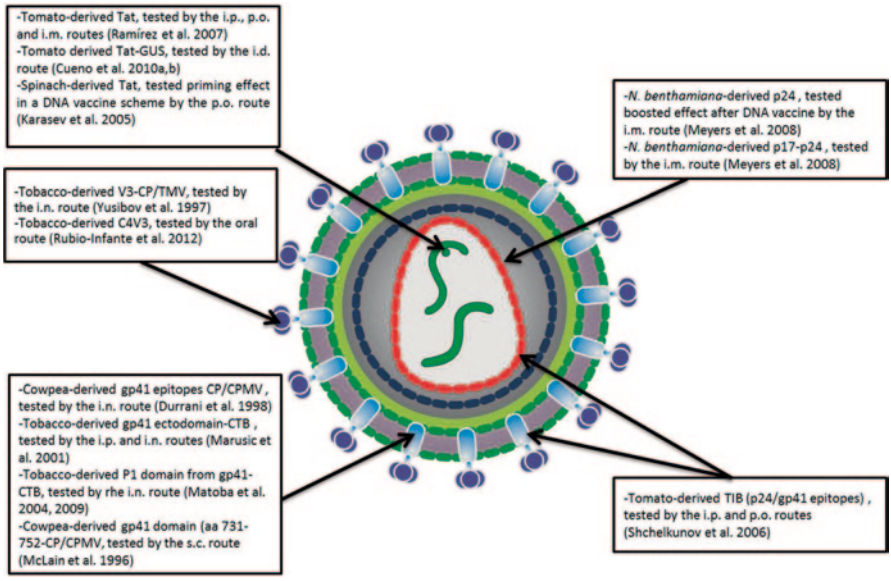

Fig. 9.1 Schematic representation of the HIV components used as targets of plant-based vaccine models resulting in immunogenic activity to date. *p.o.* oral, *i.n.* intranasal, *i.m.* intramuscular, *i.p.* intraperitoneal. *CP* coat protein, *CPMV Cowpea mosaic virus, CTB* cholera toxin B subunit, *AMV Alfalfa mosaic virus TMV Tobacco mosaic virus*

(Walker et al. 2011; Graham et al. 2010). To induce humoral immunity, the HIV-1 envelope glycoproteins (Env) complex is considered an ideal target for the induction of neutralizing antibodies (NAbs) that can block viral entry into the host cell or induce antibody-dependent cell-mediated cytotoxicity (ADCC), (Klasse et al. 2002; Pantophlet and Burton 2006). Other structural and early components, such as Gag, Tat, or Nef, have also been assessed as vaccine candidates (Mascola et al. 1999; Ferrantelli et al. 2011).

The cost of a vaccine is indeed a key factor in the development of HIV vaccines, particularly when the majority of HIV-infected people live in developing countries where large segments of the population live in poverty. Thus far, several plant-based vaccine models have been developed that can be used to assess the efficacy of a number of HIV immunogens in test animals (Fig. 9.1). As the most common routes of HIV transmission are the genitourinary and rectal mucosa, wherein HIV invades across epithelial cells (Hladik and Hope 2009), mucosal vaccines are proposed as viable candidates that can induce local mucosal immune responses that would eventually mediate immunoprotection on mucosal surfaces (Levine 2000; Lamm 1997). The following sections provide an overview of advanced models of plant-based candidate vaccines against HIV (Fig. 9.2).

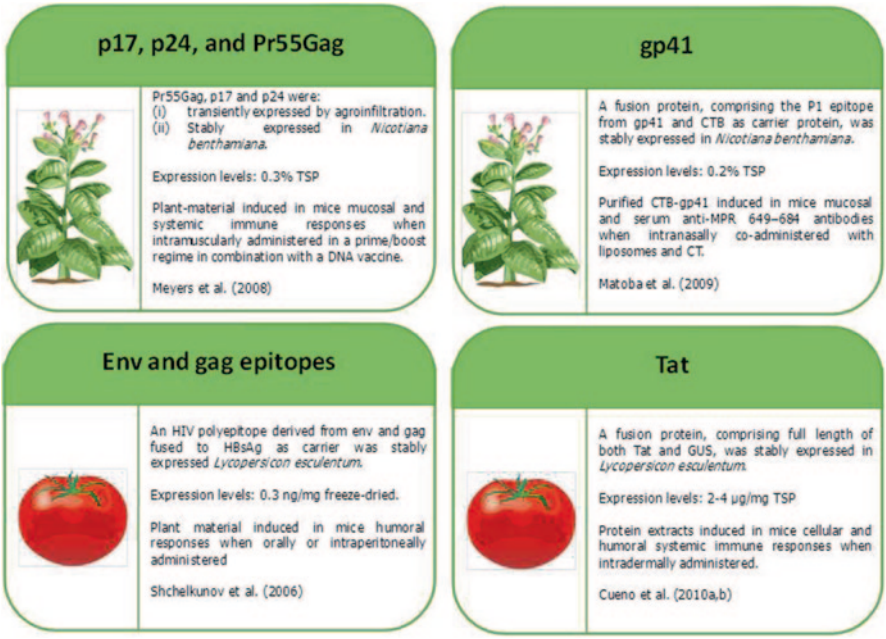

Fig. 9.2 Schematic summary of representative reports addressing plant-based vaccination against HIV components

The Gag Protein is Efficiently Expressed in Nicotiana benthamiana and Capable of Inducing Humoral and Cellular Responses

Structural HIV proteins comprise the components of the mature assembled virus particles, such as the nucleocapsid core, Gag, proteins. Pr55Gag is the protein encoded by the *Gag* gene, and it is cleaved by viral protease to yield mature Gag proteins, which consist of the matrix (p17), capsid (p24), nucleocapsid (p7), and p6 proteins. Pr55Gag has been produced in a variety of host cells leading to the assembly of highly immunogenic virus-like particles (VLPs; Deml et al. 2005; Jaffray et al. 2004). These VLPs are safe for immunization as they are non-infectious and capable of eliciting potent cellular and humoral responses (Doan et al. 2005). The p24 protein not only serves as an early indicator of HIV infection but also has been used in immunization studies as it is capable of inducing cellular and humoral responses. Two observations highlight the importance of this antigen. This core protein is the target of T-cell immune responses in both primary and chronically infected individuals; whereas, absence of anti-Gag antibodies has been associated with disease progression. These findings support the argument that p24 is a viable target for a candidate vaccine (Montroni et al. 1992; Reddy et al. 1992; Benson et al. 1999; Dyer et al. 2002; Novitsky et al. 2003). The p17 protein mediates intramembrane associations that are important in viral assembly, as well as their release and transport

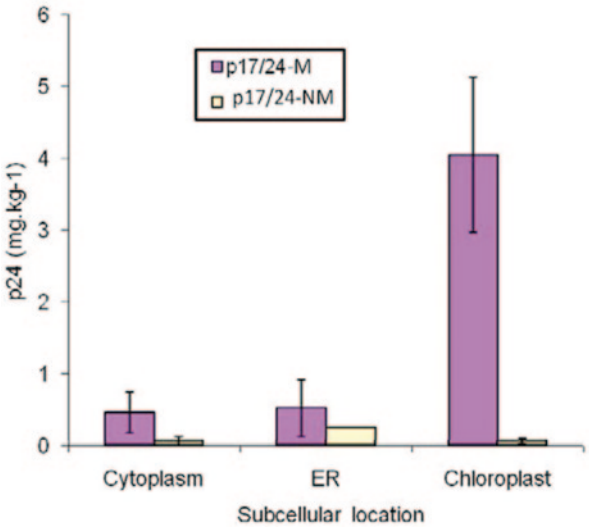

Fig. 9.3 The effect of intracellular localization on accumulation of myristilated p17/p24. Transient expression of HIV-1 p17/p24 protein in *Agrobacterium*-infiltrated *N. benthamiana* was measured by HIV-1 p24 ELISA 4 days after infiltration. (Taken from Meyers et al. 2008)

into the nucleus. Interestingly, low titers of p24 antibodies have been associated with higher incidence of AIDS. Thus, p24 is deemed as a relevant target for viral depletion (Cheingsong-Popov et al. 1991; Burkinsky et al. 1993; Novitsky et al. 2003).

In an extensive study conducted in South Africa, Meyers et al. (2008) explored the production of structural HIV antigens in *N. benthamiana* plants using both stable and transient expression systems. They utilized sorting signals to direct the heterologous protein to either the endoplasmic reticulum (ER) or to the chloroplast. In this study, the production of the HIV-1 Gag-precursor protein, Pr55Gag, as well as a truncated Gag (p17/p24), and the p24 capsid subunit were assessed. All these antigens were detected in plant tissues; however, expression levels of Pr55Gag were quite low (< 0.01 μg/kg fresh leaf). Interestingly, using an *Agrobacterium*-mediated transient expression of p24 and p17/p24 resulted in relatively higher yields, up to 4 mg/kg fresh weight (0.3 % TSP). Moreover, the expression of chloroplast-targeted proteins was higher for both p24 and p17/p24 (Fig. 9.3). Transiently expressed p17/p24 protein effectively boosted T-cell and humoral responses in mice primed with the DNA vaccine pTHGagC. Therefore, these findings suggested that these plant-produced structural HIV proteins were promising candidates for further efforts in developing plant-derived HIV vaccines (Meyers et al. 2008).

An Immunogenic CTB-P1 Protein is Produced in Nicotiana benthamiana Plants

The envelope (Env) glycoproteins reside on viral surfaces and play critical roles in pathogenesis. The Env glycoprotein gp160 serves as a precursor of gp120 and gp41 which in turn target the receptor and co-receptors, respectively. As these interactions

mediate viral entry into host cells, Env is a prime target for neutralization by antibodies, a critical step in vaccine development (Doms 2004). In particular, elements of the third variable domain of gp120 (V3) are important conformational determinants that act as binding sites to the co-receptors CCR5 and CXCR4 (Haynes et al. 2006). However, targeting the V3 loop is rather challenging due to its high frequency of mutation which leads to changes in its antigenic properties that may subsequently evade neutralizing antibodies. Interestingly, it has been reported that broad neutralizing antibody responses against Env are induced in large percentages of HIV-infected individuals (Simek et al. 2009; Stamatatos et al. 2009). Some of these Env proteins have been deemed as candidates for the development of passive immunity, while others have provided complete protection in test animal models (Burke and Barnett 2007).

Thus far, various HIV plant-based approaches based on the expression of Env epitopes have been reported. Most of these strategies rely on the use of chimeric proteins formed by a carrier such as VLPs or the cholera toxin B subunit (CTB) or a target HIV antigen. In one study, a CTB-based chimeric protein containing the V3 loop was produced in potato plants following stable transformation (Kim et al. 2004). The potato-derived CTB-gp120 was assembled into pentamers, an oligomeric form eliciting high immunogenicity, and found that it retained antigenic properties of both components. Immunogenicity of this candidate vaccine is yet to be determined (Kim et al. 2004).

In another study, Matoba et al. (2004) produced a fusion protein comprised of CTB and the P1 peptide from gp41 (aa 649–684) following transient transformation in both *N. benthamiana* and *Escherichia coli*. Both expression hosts yielded a CTB-P1 protein capable of evoking both serum IgG and mucosal IgA responses when administered intranasally to mice. This suggested that this plant-derived CTB-P1 was a promising mucosal immunogen. This *E. coli*-derived CTB-P1 was found to elicit transcytosis-neutralizing antibodies, thus offering promise as a worthy candidate for further studies (Matoba et al. 2004). Further evaluations of CTB-P1 (CTB-$MPR_{649–684}$) produced in *N. benthamiana* revealed production of mucosal and serum anti-$MPR_{649–684}$ antibodies in test mice following mucosal priming/systemic boost immunization (Matoba et al. 2009; Fig. 9.4).

Humoral and Cellular Immune Responses are Induced by a Tat Fusion Protein Produced in Tomato Plants

Early antigens refer to those proteins that are expressed during early stages of the viral replication cycle. In particular, HIV possesses six of such proteins, including Tat, Rev, Vpu, Vif, Nef, and Vpr. Of these, Nef, Tat, Vpr, and Vpu have been detected in soluble forms in sera of HIV-1-infected patients. These are likely to be released by infected/apoptotic cell, enter into macrophages, and then modulate both cellular machinery and viral transcription (Herbein et al. 2010). It is well established that Nef, a 27-kDa protein, downregulates cell surface expression of CD4, CD28, and MHC class I (Lundquist et al. 2002; Yang et al. 2002), thus exerting a

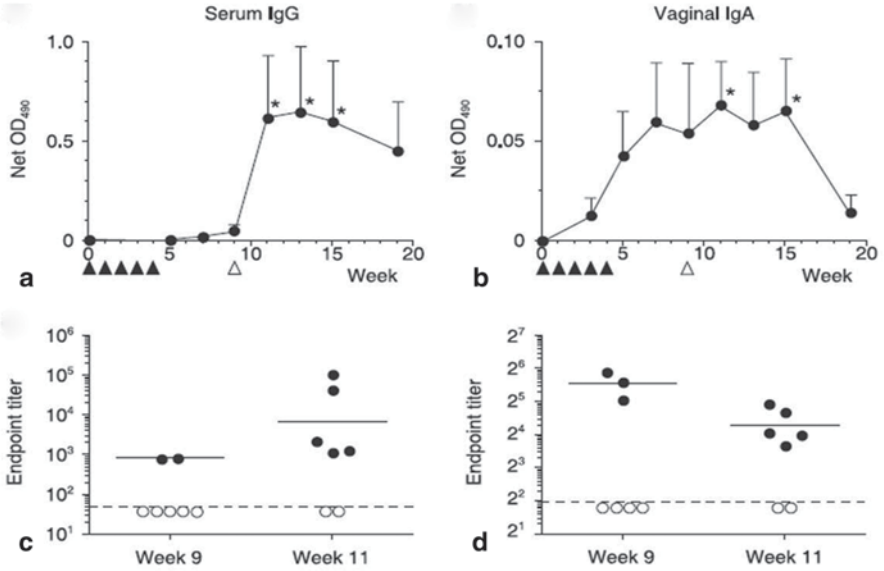

Fig. 9.4 Immunization of mice with *N. benthamiana*-derived CTB-MPR$_{649–684}$. Mice were intranasally immunized with 35 μg of liposome-conjugated CTB-MPR$_{649–684}$ and 1 μg cholera toxin (*CT*) at weeks 0, 1, 2, 3, and 4, and boosted intraperitoneally with 3 μg of the liposome-conjugated fusion protein at week 9. Immunoglobulin G (*IgG*) levels in serum samples at 1:50 dilution (**a**) and IgA levels in vaginal lavage samples at 1:4 dilution (**b**) were assayed by ELISA at the indicated weeks. Net optical density (*OD*) values are presented as arithmetic means ± standard errors of the mean. *Asterisks* indicate statistical significance when compared with week 0 ($P<0.05$, by repeated measures of analysis of variance (*ANOVA*) with Bonferroni's Multiple Comparison Test). End point titers of serum IgG (**c**) and vaginal IgA (**d**) before and after boosting (weeks 9 and 11, respectively) were determined at the reciprocal of the sample dilution factor, yielding OD values equal to averages of those from control wells (no antibodies) using extrapolation based on power series of curve fit. *Horizontal bars* correspond to geometric mean values of the responding mice (indicated by *filed circles*) at each time point. *Broken lines* correspond to detection limits of samples analyzed using ELISA (reciprocal of lower sample dilutions: 50 for serum IgG, 4 for vaginal IgA). *Open circles* under the *broken line* indicate mice that did not show any detectable response at each week and representing "non-responders." *CTB* cholera toxin B subunit, *MPR* membrane proximal (ectodomain) region of gp41. (Taken from Matoba et al. 2009)

key role in HIV pathogenesis. Interestingly, several studies have shown that Nef can induce cellular responses. Therefore, these factors justify the use of Nef as an HIV immunogen (Rolland et al. 2011; Shen et al. 2011).

Tat is a small protein that influences the transcription of viral genes and viral replication. In particular, it potently transactivates LTR-driven transcription and enhances viral gene expression (Kessler and Mathews 1992; Zhou and Sharp 1995). This HIV component has been successfully used for the elicitation of cellular responses, which along with its role in HIV replication renders it as a viable HIV immunogen (Ensoli et al. 2010).

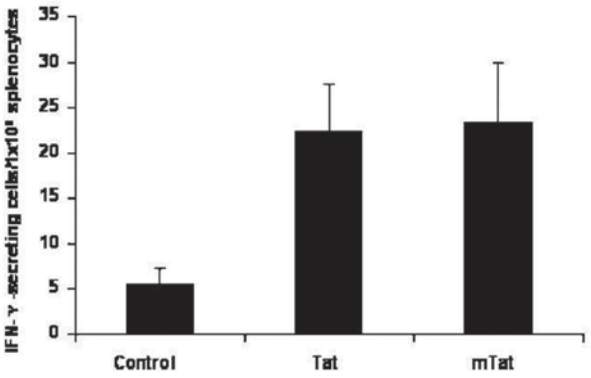

Fig. 9.5 Induction of cellular immune responses in BALB/c mice using recombinant tomato extracts. IFN-γ-secreting cells were determined by ELISPOT assay. CD8 + cells were prepared from spleens of each mouse. Approximately 5–6.25 × 10^4 cells per well were incubated with the synthetic Tat peptide (Tat 17–25) for 24 h. Plates were washed by PBS-T and further incubated overnight at 4 °C in the presence of 2 μg/ml of biotinylated anti-mouse IFN-γ monoclonal antibody, and the number of IFN-γ-secreting CD8 + T cells were visualized by adding streptavidin-conjugated alkaline phosphatases. Data correspond to means and standard deviations of three independent experiments. (Taken from Cueno et al. 2010b)

In a relatively recent effort, expression of a fusion protein comprised of Tat and GUS was investigated in tomato (Cueno et al. 2010a). Detectable levels of the expected recombinant protein were reported. When the immunogenicity of this tomato-derived recombinant protein was assessed in BALB/c mice, it was found that this candidate vaccine induced specific immune responses in mice immunized intradermally as both humoral and cellular immune responses were detected (Cueno et al. 2010a, b) (Fig. 9.5).

A Tomato-derived Chimeric Protein Comprised of gp41 and p24 Epitopes is Capable in Eliciting Humoral Responses

Considering the complexity of pathogenic HIV strains and their genetic variability, designing chimeric proteins carrying several HIV target epitopes is critical for the purpose of developing vaccines with broad and effective immune responses. When constructing a single chimeric protein comprised of a set of selected epitopes, e.g., epitopes from different isolates, it is important to exclude non-immunogenic regions that may mask presentation of the antigen to target neutralizing epitopes. In addition, sequences with adjuvant activities should be also included into the construct.

Utilizing the above strategy, a construct of a TBI multiepitopic sequence consisting of gp41 and p24 fused to the surface antigen of the hepatitis B virus (TBI-HBsAg) was generated and used to transform tomato (Shchelkunov et al. 2006).

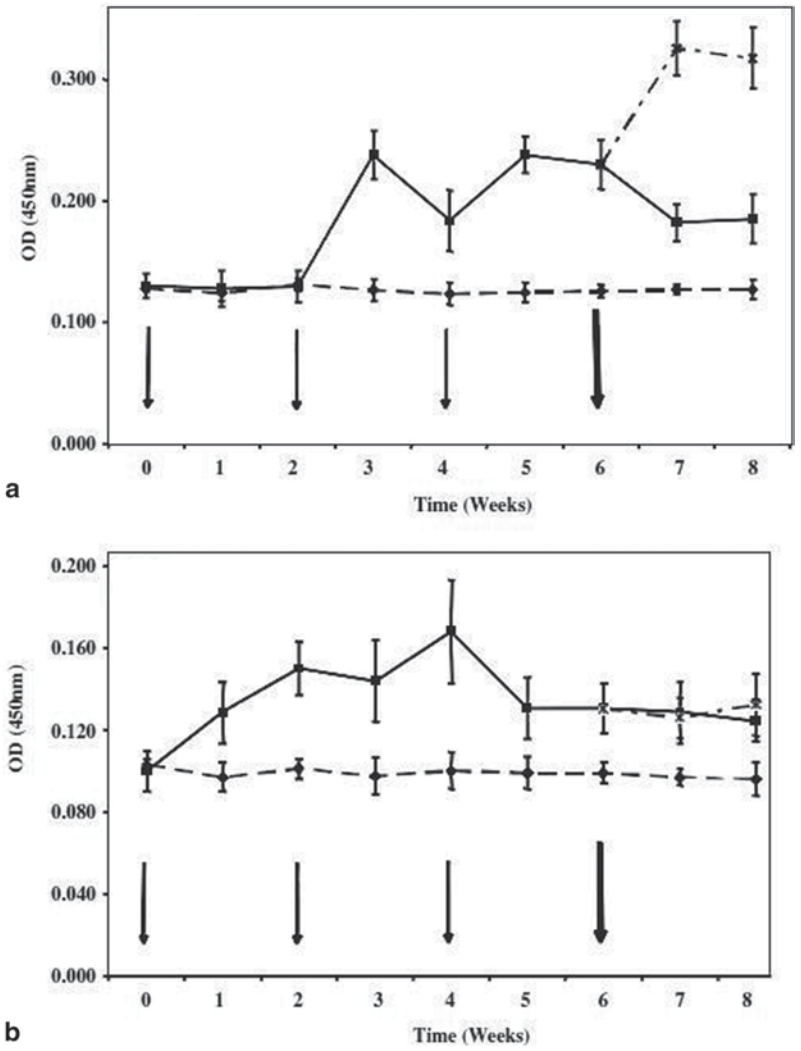

Fig. 9.6 Dynamics of anti-HIV antibody levels in (**a**) serum and (**b**) feces of mice upon feeding with transgenic and non-transgenic tomatoes and injection with a DNA vaccine: *Fine vertical arrows* indicate days when tomatoes were fed to animals; *bold short arrow* indicates immunization with a DNA vaccine. Mean values are shown and standard errors of measurement by ELISA are indicated by *bars*. (♦) non-transgenic tomato, (■) transgenic tomato, (x) DNA vaccine. (Taken from Shchelkunov et al. 2006)

Transgenic tomato plants yielded a recombinant protein capable of inducing a strong immune response when these tissues were orally administered to mice and accompanied by an intraperitoneal boost with a DNA vaccine. These findings suggested that this multiepitopic recombinant protein produced in tomato was a good candidate vaccine (Shchelkunov et al. 2006; Fig. 9.6).

Prospective View

Currently, there are no available prophylactic treatments for HIV. This is due to difficulties in developing an effective immunoprotective vaccine. However, a number of vaccine candidates are undergoing phase III clinical trials over the past few years. The most promising trial reported to date consists of administration of ALVAC, a Canarypox vector-based vaccine, and AIDSVAX, a vaccine formulated with a recombinant gp120. An efficacy of 30% for prevention of HIV acquisition has been reported in a population of 16,402 participants. This formulation is deemed safe and well-tolerated. This serves as the first vaccine with significant immunoprotective potential, thus offering new opportunities to investigate and characterize the protective immune observed in this study (Pitisuttithum et al. 2006).

With these promising results, it is important to take into consideration that vaccination costs will impact their use in global scale immunization programs. In this context, plant-based vaccines would serve as economical platforms for the production of such vaccines. As presented herein, evaluation of HIV vaccination models using transgenic plants as production platforms has made significant strides over the last two decades (Lössl and Waheed 2011). Although many models have been reported, including transient and stable expression systems using nuclear and chloroplast levels, evaluation of immunogenic properties of most candidates is still pending. Among aspects yet to be explored, immunization schemes using a priming phase by administering a parenteral vaccine and boosts, provided by plant-made vaccines, at the mucosal level would be a viable option. For example, intranasal immunization is well recognized as a route involved in the elicitation of humoral responses in genital mucosa (see Chap. 2), which would be of particular importance in preventing sexual transmission of HIV.

The assessment of chimeric proteins of several epitopes may substantially contribute to advances in this field as broad immune responses may be induced by such molecules. In a recent report by Govea-Alonso et al. (2013), a chimeric protein, C4(V3)6, comprised of the following sequences of gp120, a segment of the fourth conserved domain (C4) and six tandem repeats of the third variable domain (V3) representing distinct HIV isolates, has been synthesized. This candidate has been expressed in lettuce plants using nuclear transformation. The lettuce-derived C4(V3)6 is found to induce broad humoral responses when orally administered to mice (Govea-Alonso et al. 2013). Such multiepitopic strategies are most likely to induce broad immune responses, and thereby be successful vaccine candidates.

On the other hand, expression of gp120 in plant cells remains unexplored despite its utilization in the development of the AIDSVAX formulation used in the most effective immunization scheme design available thus far (Flynn et al. 2005). Therefore, assessing the gp120 biosynthesis capacity is an important goal that should be pursued. In addition, specific glycosylation plays an important role in antigenic and immunogenic properties of gp120-based immunogens (Mori et al. 2005; Yu et al. 2012). Although plant glycosylation differs substantially from that in mammalian systems, current technologies offer possibilities of obtaining human-like glycosylations which would serve as useful tools in the development of gp120-based vaccines produced in plant cells (Bosch and Schots 2010; Lerouge et al. 1998; Schähs et al. 2007).

In conclusion, progress has been made in the development of HIV plant-based vaccines due to the availability of a promising "proof of concept." This requires the pursuit of further experimentation and studies, along with completion of preclinical trials for reported candidate vaccines. However, it is important to continue to explore the development of more studies on the production of new immunogens that will induce strong immunoprotection against HIV/AIDS.

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Chapter 10
Plant-Based Vaccines Against Hepatitis B

Tomasz Pniewski

Abbreviations

AlMV	*Alfalfa mosaic virus*
BeYDV	*Bean yellow dwarf virus*
CaMV 35S promoter	Promoter of 35S RNA of cauliflower mosaic virus
CHO	Cell line derived from Chinese hamster ovary
CLPs	Capsid-like particles
CPMV	*Cowpea mosaic virus*
CTB	Cholera toxin subunit B
DW	Dry weight
EFE	Ethylene forming enzyme
ER	Endoplasmic reticulum
FW	Fresh weight
GALT	Gut-associated lymphoid tissue
GMP	Good manufacture practice
HAV	Hepatitis A virus
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HDV	Hepatitis D (delta) virus
HBcAg	Hepatitis B core Antigen
HBsAg = HBs antigen(s)	(any) Hepatitis B surface Antigen(s)
rHBsAg	Recombinant Hepatitis B surface Antigen
S-, M-, L-HBsAg	Small, medium or large HBsAg
HCC	Hepatocellular carcinoma
HepB	Hepatitis B
HIV	*Human immunodeficiency virus*
i.m.	Intramuscular
i.n.	Intranasal

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i.p.	Intraperiteoneal
LT-B	Heat-labile enterotoxin subunit B
MALT	Mucosa-associated lymphoid tissue
MAS	Mannopine synthase
mIU/ml	Milli-international unit/ml = unit of titer of anti-HBs antibodies
NOS	Nopaline synthase
OCS	Octopine synthase
PVX	Potato virus X
S-IgA	Secretory IgA
SVP	Subviral particle
TEV	Tobacco etch virus
TSP	Total soluble protein
5′-UTR	5′-untranslated region of mRNA
VLPs	Virus-like particles

Introduction

A vaccine against hepatitis B (HepB), or hepatitis B virus (HBV) as the etiology pathogen, occupies a particular place among both plant-based and other types of vaccines. A yeast-derived vaccine, based on the small surface antigen of HBV (S-HBsAg), was the first subunit vaccine. Recombinant anti-HBV vaccines, containing S-HBsAg and/or other virus antigens, belong to the most efficacious specimens and facilitated mass-scale prophylaxis programs against one of the major human diseases. The small surface antigen was also the first one produced in plants and subsequently used as a prototype injection vaccine. Similarly as plants bearing heat-labile enterotoxin B subunit (LT-B) or cholera toxin B subunit (CTB), those with S-HBsAg were investigated at the very beginning of research on edible vaccines. However, in the latter case, it was the first attempt to orally induce immune response against a blood-borne pathogen. Up to date, a vaccine against HepB is one of the most investigated plant-based ones. HBV antigens, mainly S-HBsAg, were expressed in many plant systems and examined in a number of trials on oral and parenteral immunization. The 20-year story of plant-based vaccines against HepB, their successes and disappointments, is a prime example of evolution in opinions on such vaccines as a whole. Although original assumptions of anti-HBV plant-based vaccines have been revised gradually, gained knowledge and technologies are invaluable. Therefore, the idea of plant-derived anti-HBV vaccines is still vital and new approaches can be proposed today.

Epidemiological Impact of Hepatitis B

A liver disease called jaundice has been known since early antiquity, but blood transmission of most hepatitis forms was documented as late as in 1885. A viral origin of the disease was recognized in the late 1930s (Hollinger 1996) and HBV

was identified in the 1960s (Blumberg et al. 1965). HepB is one of the diseases characterized by the most complex course, the highest morbidity and mortality. Pathogenesis of HepB is extraordinarily intricate and depends on immune system functioning and the capability of the virus to avoid immune response (Hilleman 2003). The HBV infection may lead to the unapparent (65–80 % of cases) or acute (20–35 %) form of the disease, when patients require hospital care. Sometimes, fulminant hepatitis (0.1–1 %) develops, which terminates in death (Hollinger 1996). Progressive liver dysfunction in active HepB causes nausea and vomiting, fever, fatigability, myalgia, weight loss, and jaundice. Although spontaneous complete eradication of HBV occurs in ≥90 % of cases, 7–10 % of patients become carriers of the virus. However, chronic HepB morbidity depends on the patient's age and the corresponding competence of the immune system. Among infected persons, approximately 90 % of newborn babies, 50 % of infants, and 30 % of children, but only 5–10 % of adults, become HBV carriers (Hilleman 2003). Chronic HepB can adopt various forms, from silent, through persistent with remissions and exacerbations, to liver fibrosis, cirrhosis, and hepatocellular carcinoma (HCC). HBV accounts for the etiology of 55 % HCC cases, which is among the first ten most common cancers in the world—ca. 600,000 new cases per year, and is the predominant malignant tumor of the liver (Kew 2010). Overall, persistent HepB leads to death in 20–25 % virus carriers (Hilleman 2003). In addition to their sufferings, chronic carriers comprise a reservoir of the virus.

HepB is still one of the most common human diseases. One third of the global population still lives in regions with a high risk of HBV infection, yet prevalence of the virus is uneven. Nowadays, North and South America (except for some endemic areas), Australia, and Western Europe are regions where HBV prevalence dropped, i.e., < 1 % of the population and exposure rate is below 2 %. In regions of intermediate HBV prevalence, such as Central and Eastern Europe, the Mediterranean region, the Middle East, the Indian subcontinent, and Japan, chronic carriers account for 1–7 % of the population and the risk of infection is 10–60 % (Kew 2010; Shepard et al. 2006; WHO Media Centre 2012; Hilleman 2001; Romano et al. 2011; Michel and Tiollais 2010). However, in the sub-Saharan Africa, Central and Southeastern Asia, and China, 70–90 % of the population is exposed to the virus, and prevalence reaches 8 % or more. Despite mass immunization programs, the number of chronic carriers worldwide is slowly, but steadily, growing (Kew 2010; Shepard et al. 2006; WHO Media Centre 2012; Hilleman 2001; Romano et al. 2011; Michel and Tiollais 2010). It has increased from 250 million in the late 1980s, when the vaccination programs against HepB were implemented, up to around 400 million at present.

Extraordinary HBV infectivity and high stability in the external environment determine its high prevalence. The virus easily and rapidly spreads through blood or other body fluids, even when found in trace amounts (Hollinger 1996; Hilleman 2001). Only 0.1 μl of infected blood is sufficient to evoke HepB (Hilleman 2001). The virus spreads in many ways. In developing countries of Asia, HBV is mostly transmitted at childbirth and lactation, while in the sub-Saharan Africa the virus spreads mainly by horizontal transfer during early childhood infections, sexual contacts, unsafe injections and transfusions, and also some rituals. In turn, a majority of infections in developed countries occur among adolescents or young adults during

sexual activities or intravenous narcotic use (Hilleman 2003; Kew 2010; Shepard et al. 2006; WHO Media Centre 2012).

Overall, approximately 4.5 million new HBV infections are recorded worldwide each year. From among those, 15–40 % patients develop hepatic failure or chronic hepatitis, including cirrhosis and HCC. As it is estimated, mortality from acute, fulminant, and chronic HepB or post-disease complications reaches 600,000 to 1 million people every year (Kew 2010; WHO Media Centre 2012; Romano et al. 2011; Michel and Tiollais 2010). Moreover, HepB substantially increases the risk of hepatitis D (delta) virus (HDV) or hepatitis C virus (HCV) superinfection or coinfection by HIV (Mallet et al. 2011; He et al. 2011) or other dangerous viruses. All these facts result in a situation when controlling HBV continues to be one of the most vital goals of medicine, both in terms of therapy and prevention.

It may be stated that therapy of chronic HepB using interferons or nucleotide analogues and other drugs has essentially progressed (Deres et al. 2003). However, it is still not completely efficacious or remains too expensive for most developing, i.e., also the most threatened countries (Michel and Mancini-Bourgine 2005; Yuen and Lai 2011). The cost of therapy using interferon alpha (IFN-α), or some inhibitors of viral polymerase such as adefovir, reaches US$ 12,000–15,000/person year. Other drugs are much cheaper, e.g., lamivudine up to US$ 1,500; however, they are often effective only to a limited extent or their long-term usage may lead to side effects, such as emergence of new drug-resistant HBV serotypes, etc. (Michel and Mancini-Bourgine 2005; Yuen and Lai 2011; Liaw et al. 2004). Therefore, although new drugs and therapeutic vaccines against chronic HepB have been intensively investigated, prevention—first of all through vaccination—still remains the most important and practical way to restrain HBV expansion.

The first-generation vaccines against HepB were introduced in the early 1980s (Krugman 1982) and contained subviral particles (SVPs) of HBV, purified from the inactivated serum of carriers. The vaccines, e.g., Hepatavax-B (Merck & Co., New York, NY, USA) or Hevac B (Pasteur, France), were highly efficacious, but were costly and the produced quantities were inadequate to meet the needs. These reasons, together with concerns about safety of blood products, enforced research on a new type of vaccine (Shouval 2003). In the early 1980s, the invention of subunit vaccines founded on the main HBV antigen—S-HBsAg, recombined in yeast (rHBsAg), was an absolute milestone in HepB prevention (McAleer et al. 1984). These vaccines were practically as effective as the first-generation vaccines, but much cheaper. It is thanks to the implementation of the subunit vaccines into mass immunization programs that the HBV prevalence has become partly controlled and the number of chronic carriers has declined in many countries, as mentioned above, especially in industrialized or rapidly developing ones (Kew 2010; WHO Media Centre 2012; Romano et al. 2011; Michel and Tiollais 2010). Currently, many vaccines are commercially available or are being tested, including plasma-derived and subunit vaccines, produced in yeast and mammalian cells (Romano et al. 2011; Michel and Tiollais 2010; Michel and Mancini-Bourgine 2005; Shouval 2003; Brocke et al. 2005). All of them are administered intramuscularly and contain a single or several HBV antigens.

Vaccination Targets in Hepatitis B Subunit Vaccines

All structural proteins of HBV are strong antigens utilized as key components of different vaccines. The virion (Dane's particle) is 42 nm in diameter and consists of the nucleocapsid and the envelope. The capsid is assembled from the basic C protein known also as the core antigen (Hepatitis B core Antigen, HBcAg, 183–185 aa, 21 kDa). The envelope is constituted by a membrane coming from the endoplasmic reticulum (ER) of the host cell and three embedded proteins—subunits of the surface antigen (Hepatitis B surface antigen, HBsAg)—small (S, 226 aa, 24 kDa—p24), medium (M, 281 aa, 31 kDa—p31), and large (L, 389–400 aa, 39 kDa—p39). The subunits are often referred to as individual surface antigens—S-, M-, and L-HBsAg. The surface proteins are encoded by a common gene with three autonomous start codons within the same reading frame. Thus, all proteins contain the common and the largest domain S, which alone consists of S-HBsAg and carries additional preS domains at its N-terminus—preS2 in the case of M-HBsAg, while L-HBsAg has preS2 and preS1. The S domain/protein is the main structural component of the envelope. It is a highly hydrophobic polypeptide with four subdomains anchoring the whole molecule in envelope lipids, but it also contains a large, hydrophilic, subdomain orientated to the outside with immunodominant epitope "a" (Ganem 1996; Bruss 2004, 2007). PreS domains play an essential role in virion assembly and entry to the hepatocyte during infection (Bruss and Vieluf 1995; Glebe and Urban 2007). Approximately 50 % molecules of surface antigens are N-glycosylated in the S corpus and for M/L-HBsAg also in the preS domains. Glycosylated S-, M-, and L-HBsAg proteins are denoted as p27, p33, and p42 (or gp27, gp 33, and gp42), respectively, due to their higher molecular mass. M-HBsAg can also be *O*-glycosylated and L-HBsAg is mirystylated at the N-terminus of the preS1 domain. Taken as a whole, forms of S-, M-, and L-HBsAg comprise >80–95 %, 5–15 %, and 1–2 % of envelope proteins, respectively (Ganem 1996; Bruss 2004, 2007). HBsAg and HBcAg molecules once synthesized dimerize by disulfide bonds. Dimers are basal structural units of virions and SVPs.

The particular feature of HBV antigens is their self-assembly in the absence of genomic DNA into SVPs—capsid-like particles (CLPs) or virus-like particles (VLPs). SVPs are much stronger immunogens than soluble, individual, or dimerized antigens, due to their multiplication and increased durability. Moreover, SVPs are absolutely safe as non-infectious DNA-free structures, but resemble virions and induce immune response directed to the virtual virus. These facts, together with the capacity of manufacturing SVPs in different recombinant expression systems, such as yeast, mammalian cells, etc., are the foundations for progress in anti-HBV subunit vaccines. CLPs consist of 90–120 molecules of HBcAg organized in spherical structures of 30–34 nm in diameter (Bruss 2004). VLPs emerge naturally for the duration of HBV infection, overproduced up to 10,000-fold relative to virions, probably as dummies for the host's immune system. VLPs consist mostly of S-HBsAg itself or with a portion of other surface antigens, structured into spheres of 20 nm in diameter and around 100 HBsAg molecules or filaments of the same diameter, but with variable length. VLPs obtained in recombinant systems are only spherical,

but yeast-derived particles contain non-glycosylated HBsAg. Recombinant VLPs consisting of S-HBsAg and other surface antigens, as well as formed solely by M-HBsAg, can also be obtained (Bruss 2007). Capability of L-HBsAg to form VLPs is limited, although VLPs formed in some cell cultures solely by L-HBsAg have been reported (Yamada et al. 2001). Yet, VLPs containing the L-antigen can be produced mainly by co-expression of S- and L-HBsAg (Brocke et al. 2005), as well as assembled by the chimeric antigen, where the preS1 domain was fused directly to S-HBsAg (Yang 2000).

The role of S-HBsAg in VLP formation and functioning predestined this antigen as the foundation for prophylactic subunit vaccines against HepB. Most of those belong to the group of the second-generation vaccines, e.g., Engerix B® (GlaxoSmithKline, Belgium), Recombivax HB® (Merck & Co., New York, NY, USA), HBVax (Pasteur, France) and many others, formulated by VLP-assembled S-HBsAg, usually adjuvanted with alum hydroxide. The technology of gene recombination in yeast (*Saccharomyces cerevisiae, Hansenula polymorpha, Pichia pastoris*) combined with bioreactor processing facilitated low-cost commercial-scale vaccine production. Subunit vaccines based on S-HBsAg meet safety expectations and are highly effective; 90–95 % of immunized patients produce specific anti-HBs antibodies up to a titer ≥ 100 mIU/ml, while 10 mIU/ml has been adopted as sufficient protection (International Group 1988). The above-mentioned vaccines were put into clinical practice for the first time in the late 1980s (Romano et al. 2011; Michel and Tiollais 2010; Shouval 2003; Brocke et al. 2005). Since then, together with later generics, they have been commonly used for almost 30 years as a basic tool in HepB prevention.

However, it was revealed some years after the implementation of the second-generation vaccines, that despite their exceptional efficacy, there are some groups of nonresponders, especially the elderly, the obese, patients with immunodeficiency syndromes or other ailments, as well as individuals probably genetically predisposed (Shouval 2003; Alper et al. 1989; Singh et al. 2003). Moreover, mass vaccinations increased selection pressure (Cooreman et al. 2001; Huang et al. 2004), which promoted mutated HBV strains with S-HBsAg altered within the neutralizing "a" epitope, thus resistant to antibody attack.

Hence, since the late 1980s, many research projects have been conducted to develop more efficacious vaccines for prevention purposes. Two approaches were adopted. The first one seemed obvious—to elaborate classical vaccines, but enclosing S-HBsAg from newly appearing HBV strains. The other one was connected with the generation of novel, third-generation vaccines which would include, alongside S-HBsAg, the other envelope proteins of HBV, i.e., M-HBsAg and/or L-HBsAg. These antigens induce a larger spectrum of anti-HBV antibodies, as their characteristic domains preS1 and/or preS2, apart from their functions, are strong immunogenic determinants displayed on a virion or SVP surface. Consequently, vaccines containing two or all the three HBV surface antigens exhibited an enhanced immunogenicity (Shouval et al. 1994; Zuckerman et al. 1997; Young et al. 2001). However, expression of native M- and especially L-HBsAg in microorganisms, albeit possible (Brocke et al. 2005; Han et al. 2006), is still not routine. These antigens

are usually produced in Chinese hamster ovary (CHO) and other mammalian cell expression systems, exceptionally in yeast systems (Shouval 2003). Therefore, although third-generation vaccines such as GenHevac (Pasteur, France), Hepacare® (Medeva Pharma Plc, UK), and Bio-Hep-B™ (Bio-Technology General, Israel), and others have been gradually introduced into practice (Madaliński et al. 2002; Rendi-Wagner et al. 2006), due to their high cost, they are still not as commonly administered as conventional second-generation vaccines, and remain restricted mainly to special cases, such as nonresponders to S-HBsAg-based vaccines, etc.

The importance of M- and L-HBsAg in the control of HepB results from the fact, that apart from prophylaxis, these antigens together with S-HBsAg have also been considered as components of postulated therapeutic vaccines for HBV chronic carriers (Michel and Tiollais 2010; Michel and Mancini-Bourgine 2005). The rationale of using specimens functioning by a "vaccine mode" for medication was that rapid seroconversion and strong stimulation of T cells during immune response are crucial stages in natural HepB recovery. Nonetheless, the main reason for the application of vaccines in therapy resulted from the expense and limited effectiveness or dangerous side effects of treatments based on IFN-α or nucleotide derivatives (Michel and Mancini-Bourgine 2005; Yuen and Lai 2011; Liaw et al. 2004). In fact, "triple" vaccines proved to be effective for protecting babies born to infected mothers (Lin et al. 2003). Such specimens also increased immune response and suppression of HBV replication when delivered to adult chronic carriers (Michel and Tiollais 2010; Michel and Mancini-Bourgine 2005; Couillin et al. 1999; Pol and Michel 2006). However, despite some therapeutic potential, this effect turned out to be transient.

Therapy of chronic HepB can probably be improved by a combination of a vaccine containing HBV surface antigens with proteins or compounds enhancing or synergistic to their activity, for instance, drugs, immunoglobins, immunomodulators, etc. (Michel and Tiollais 2010; Michel and Mancini-Bourgine 2005). One of those approaches is to exploit the core HBV antigen. HBcAg is known to act as a strong adjuvant to HBsAg and activator of T cells (Böcher et al. 2001; Lobaina et al. 2005). Moreover, it can be efficiently expressed not only in yeast (e.g., *P. pastoris*) and other eukaryotic systems but also in *Escherichia coli* (Watelet et al. 2002; Li et al. 2007). In therapy of chronic HepB, unmodified HBcAg can be composed with all HBV surface antigens into one integral vaccine. Some studies showed that immune response induced initially by HBcAg led to recovery of chronic HepB (Lau et al. 2002). Another solution is to use HBcAg as a carrier for immunodominant epitopes of HBsAg proteins. The particular structure of HBcAg-assembled CLPs, where 2-nm wide spikes formed by HBcAg helixes protrude 2 nm from the CLP surface (Böttcher et al. 1997; Uetrecht et al. 2008), facilitate conjugation of relatively large protein fragments, increasing manifold their immunogenicity (Kratz et al. 1999; Vogel et al. 2005). The antiviral activity of the preS epitope fused to HBcAg confirmed the therapeutic potential of preparations founded on HBcAg CLPs (Chen et al. 2004). Nevertheless, therapy of chronic HepB using HBV vaccines remains extremely complex and is still under research.

For years, HBV antigens have been extensively investigated as subunit vaccines. Today, there are at least 40 anti-HBV vaccines, therein 3 plasma derived, around 20 subunit second-generation vaccines, and 6 third-generation subunit vaccines, as well as 10 or more polyvalent vaccines against HepB and other diseases, including bivalent vaccines against HBV and hepatitis A virus (HAV) (Twinrix™, GlaxoSmithKline) or hepatits C virus (HCV) (tested V-5 Immunitor) (Michel and Tiollais 2010; Michel and Mancini-Bourgine 2005; Shouval 2003; Brocke et al. 2005; CT Database 2013; DrugsUpdate 2013). Vaccines based on S-HBsAg are the foundation for prophylaxis against HepB, while vaccines containing S-HBsAg and other surface antigens supplement vaccination for particularly needy patient groups. However, despite those above-mentioned unquestionable successes, globally HepB, especially its chronic form, persists as a major human disease. Multilane ongoing actions are being taken to provide better effectiveness of vaccination, from improvement of accessibility to further development of vaccines. Current vaccines, but with novel adjuvants or delivery/release systems, recombinant viruses, or bacteria-bearing HBV antigens, DNA vaccines (Brocke et al. 2005), etc., as well as plant-based vaccines, are considered as candidates for new vaccines.

Plant-Based Vaccines Developed Against Hepatitis B

Background and Prelude

In the early 1990s, subunit vaccines were commercially available for barely a few years. The alarming HBV prevalence at that time and 250,000 new infections per year caused concern about the success of the recently implemented vaccination programs. The initial price of a vaccine dose—ca. US$ 40—and at the same time the three-dose immunization schedule required for complete protection generated an economical barrier for the most vulnerable, but poor countries. Furthermore, logistic problems with common access to vaccines often resulted in an uncompleted procedure and deficient protection. Under those circumstances, research on another vaccine, effective but inexpensive and commonly available, was particularly desirable.

Plant-based vaccines were supposed as a remedy to that situation. HBV antigens produced in plants were considered as alternatives, or at least supplements to injection vaccines derived from yeast or mammalian cells. Plant production was assumed to be as low cost as microbial bioreactors and much cheaper than in mammalian cells. Yet, oral immunization was thought to be the principal milestone. The most daring concepts suggested that plants bearing antigens would be utilized directly as edible vaccines (Lam and Arntzen 1996; Langridge 2000). The oral administration brought about some crucial benefits, such as reduction or elimination of vaccine processing and needle-free administration. What is more, plants as vaccine producers and carriers seemed to exhibit additional advantages, e.g., safety—due to the natural exclusion of microbial toxins or human and animal pathogens, simple

distribution, and storage at ambient temperature in the form of tubers, grains, dried fruits, which would make it possible to eliminate the cold chain, etc. Generally, plant-based anti-HBV vaccines, especially oral ones, were postulated to be cheap in production, distribution, and application, hence easily accessible, particularly for developing countries.

In fact, a vaccine against HepB soon became a leading project for the idea of plant-based vaccines. The first-ever plant expression of an antigen important for human vaccination was reported for the small surface antigen of HBV (Mason et al. 1992). That antigen was later purified and used for injection (Thanavala et al. 1995). Soon, the concept of edible vaccines was proposed, though antigens characteristic of pathogens invading the gut were studied at first—LT-B of *E. coli* and CTB of *Vibrio cholerae* (Haq et al. 1995; Mason et al. 1998; Arakawa et al. 1998). Yet, S-HBsAg was again the first antigen coming from a blood-borne pathogen which was examined for oral immunization (Kapusta et al. 1999). Since that time, all HBV antigens have been produced in plants and used for various vaccination trials (Tables 10.1, 10.2, 10.3, and 10.4).

Production of HBV antigens in plant systems

Production of HBV antigens in plants has been realized in a number of projects. Different approaches were investigated to increase its capacity, including expression systems—stable or transient, plant hosts, promoters, signal sequences, modification of an antigen coding sequence, etc. Some details are presented in Tables 10.1, 10.2, 10.3 and 10.4, but general remarks are presented below.

Among HBV antigens, S-HBsAg as the basic HBV immunogen was the main subject of research. Recently, interest in HBcAg has noticeably increased as well. Expression of M-HBsAg was reported several times, while L-HBsAg would need to be more intensively studied. In a majority of projects, the antigens were stably expressed in transgenic plants (Lam and Arntzen 1996; Mason et al. 1992; Kapusta et al. 1999; Rukavtsova et al. 2003; Sunil Kumar et al. 2006b, 2006a, 2003a, 2003b, 2005b, 2005a; Ehsani et al. 1997; Shulga et al. 2004; Zhao et al. 2000; Chen et al. 2002; Peng et al. 2002; Liu et al. 2005; Pniewski et al. 2006, 2011, 2012; Imani et al. 2002; Ganapathi et al. 2007; Kapusta et al. 2001; Kostrzak et al. 2009; Richter et al. 2000; Kong et al. 2001; Mason et al. 2003; Gao et al. 2003; Thanavala et al. 2005; Hayden et al. 2012a, 2012b; Pniewski 2012; Dogan et al. 2000; Smith et al. 2000; Sojikul et al. 2003; Huang and Mason 2004; Huang et al. 2005; Salyaev et al. 2007; Salyaev et al. 2010; Joung et al. 2004; Youm et al. 2007; Lou et al. 2007; Qian et al. 2008; Tsuda et al. 1998). Tobacco (*Nicotiana tabacum* and *Nicotiana benthamiana*), potato, and tomato (Lam and Arntzen 1996; Mason et al. 1992; Thanavala et al. 1995, 2005; Rukavtsova et al. 2003; Sunil Kumar et al. 2006b; Ehsani et al. 1997; Shulga et al. 2004; Zhao et al. 2000; Srinivas et al. 2008; Kostrzak et al. 2009; Richter et al. 2000; Kong et al. 2001; Mason et al. 2003; Gao et al. 2003; Dogan et al. 2000; Huang and Mason 2004; Huang et al. 2005, 2008;

Table 10.1 Progress of prototype plant-based vaccines based on S-HBsAg

Plant host	Maximum antigen level	Expression system[a], plant material processing	Immunization studies—subject, schedule, and results	References
Plant expression only				
Tobacco	2.9 µg/g DW	Transgenic plants	–	Rukavtsova et al. (2003)
Tobacco	19.4 ng/g FW	Transgenic plants	–	Sunil Kumar et al. (2006b)
Potato	80 ng/mg TSP	Transgenic plants	–	Ehsani et al. (1997)
Potato	1.6 µg/g FW	Transgenic plants	–	Shulga et al. (2004)
Tomato	8 µg/g FW	Transgenic plants	–	Zhao et al. (2000)
Tomato	490 ng/g DW	Transient expression using regular transformation vectors	–	Srinivas et al. (2008)
Peanut	0.032 % TSP	Transgenic plants	–	Chen et al. (2002)
Laminaria	6.67 µg/g FW	Transgenic plants	–	Peng et al. (2002)
Ginseng	184 ng/g FW	Transgenic plants	–	Liu et al. (2005)
Potato hairy roots	97.1 ng/g FW	Transgenic plants	–	Sunil Kumar et al. (2006a)
Lupin callus	6 µg/g FW	Transgenic plants	–	Pniewski et al. (2006)
Carrot cell suspension culture	25 ng/g FW	Transgenic plants	–	Imani et al. (2002)
Tobacco cell suspension culture	31 µg/l	Transgenic plants	–	Sunil Kumar et al. (2003a, b, 2005b)
Soybean cell suspension culture	700 ng/g FW	Transgenic plants	–	Ganapathi et al. (2007)
Exclusively oral immunization at priming and boosting using edible vaccines or plant-derived formulations				
Lettuce, lupin callus	35 ng/g FW 140 ng/g FW	Transgenic plants	Mice and humans, 2 × fed with raw plant tissues, ca. 0.7–1 µg S-HBsAg/dose in 1–2 mo. intervals. Anti-HBs in serum, maximum 18 mIU/ml	Kapusta et al. (1999), (2001)

Table 10.1 (continued)

Plant host	Maximum antigen level	Expression system[a], plant material processing	Immunization studies—subject, schedule, and results	References
Tobacco	ca. 10 µg/g FW	Transgenic plants. Leaf lyophilization, total S-HBsAg 103 µg/g	Mice intragastrically 2–3 × delivered lyophilized tissue, 0.5–100 ng/dose, 1–2 mo. intervals. Low titer of anti-HBs IgG and IgA in serum and S-IgA in intestine. Stimulation of Treg lymphocytes	Kostrzak et al. (2009)
Lettuce	20 (mean)—60 (maximum) µg/g FW	Transgenic plants. Leaf lyophilization, S-HBsAg VLPs 11 µg/g tablets made from lyophilized tissue with definite S-HBsAg dose, 2.3 µg/unit	Mice intragastrically 2 × delivered of lyophilized tissue, 100 ng S-HBsAg/dose, 1–2 mo. intervals. anti-HBs in serum, maximum 19 mIU/ml, and S-IgA in feces	Pniewski et al. (2011)
Combined immunization via injection and edible vaccines				
Potato	16 µg/g FW	Transgenic plants	Mice orally primed (3 × feeding/2 w., 5.5 µg S-HBsAg/dose with 10 µg CTB) and i.p. boosted (0.5 µg rHBsAg) after 10 weeks. Anti-HBs in serum, maximum 1,680 mIU/ml	Richter et al. (2000)

Table 10.1 (continued)

Plant host	Maximum antigen level	Expression system[a], plant material processing	Immunization studies—subject, schedule, and results	References
Potato, tobacco cell suspension culture	8.35 µg/g FW 16 µg/g FW 261 ng/mg TSP	Transgenic plants. Extraction and purification of the antigen	Mice orally primed (3 × feeding/2 w., 42 µg S-HBsAg/dose with 10 µg CTB) and injection boosted (0.5 µg rHBsAg) after 6–16 w., or reverse order Anti-HBs IgM and IgG in serum, maximum 3,500 mIU/ml. Anti-HBs S-IgA in intestinal mucus and saliva	Kong et al. (2001); Mason et al. (2003)
Cherry tomato	240 ng/g FW	Transgenic plants	Mice orally primed (feeding 4 weeks every day 1 µg S-HBsAg/dose) and i.m. boosted (0.5 µg rHBsAg) or i.m. primed (2 µg rHBsAg) and orally boosted as above, after ca. 20 w. Anti-HBs in serum (only according to second schedule)	Gao et al. (2003)

Table 10.1 (continued)

Plant host	Maximum antigen level	Expression system[a], plant material processing	Immunization studies—subject, schedule, and results	References
Potato	16 µg/g FW	Transgenic plants	Humans with maintained anti-HBs antibodies, previously immunized using commercial vaccines orally boosted by tuber consumption (ca. 0.8–1 mg of SHBsAg/dose). anti-HBs IgG in serum, maximum. 5,000 mIU/ml	Thanavala et al. (2005)
Combined immunization via injection and plant-derived oral formulations				
Maize	71 µg/g FW	Transgenic plants Oil extraction from grains and obtained pulp converted into pellets	Mice i.p. primed (0.5 µg rHBsAg) and orally boosted (3 × within 2 w., > 100 µg S-HBsAg/dose with 25 µg LT-B) after 15 w. Anti-HBs in serum, max. 4630 mIU/ml. Anti-HBs S-IgA in feces	Hayden et al. (2012a, 2012b)
Lettuce	Ca. 20–60 µg/g FW	Transgenic plants. Leaf lyophilization, S-HBsAg VLPs 20–25 µg/g	Mice i.m. primed (0.5 µg rHBsAg) and orally boosted after 6 and 12 w., 200 ng S-HBsAg/dose. Anti-HBs in serum, maximum 800 mIU/ml. Anti-HBs S-IgA not detected	Pniewski (2012)

Table 10.1 (continued)

Plant host	Maximum antigen level	Expression system[a], plant material processing	Immunization studies—subject, schedule, and results	References
Antigen purification				
Tobacco	65 ng/mg TSP	Transgenic plants. Extraction, immunoaffinity chromatography, sucrose and CsCl gradient ultracentrifugation. VLPs observed in TEM	–	Mason et al. (1992)
Potato	6–8 µg/g FW	Transgenic plants. Extraction, sucrose gradient ultracentrifugation. Ca. 0.8 of total S-HBsAg and 0.4 ng VLPs/µl of final preparation	–	Dogan et al. (2000)
Banana	38 ng/g FW	Transgenic plants. Extraction, CsCl gradient ultracentrifugation. Ca. 0.6 ng of total S-HBsAg/µl of final preparation	–	Sunil Kumar et al. (2003a), (2005a)
Suspension cell culture of tobacco and soybean	8 µg/g FW 74 µg/g FW	Transgenic plants. Extraction, sucrose gradient ultracentrifugation. Up to 3 ng VLPs/µl of final preparation. VLPs observed in TEM	–	Smith et al. (2002)

Table 10.1 (continued)

Plant host	Maximum antigen level	Expression system[a], plant material processing	Immunization studies—subject, schedule, and results	References
Immunization using purified antigen				
Tobacco, tomato	65 ng/mg TSP	Transgenic plants. Extraction, ultrafiltration, sucrose gradient ultracentrifugation, column concentration. Up to 100 ng of total S-HBsAg/µl of final preparation	Mice immunized i.p. 3 ×per 2 w. with plant-derived antigen (0.5 µg/dose) with Freund's adjuvant. Anti-HBsAg IgM, IgA and IgG in serum	Thanavala et al. (1995)
Tobacco cell suspension culture	226 ng/mg TSP	Transgenic plants. Extraction, sucrose gradient ultracentrifugation, column concentration. Up to 2.5 ng VLPs/µl of final preparation	Mice immunized i.p. at day 0 and 10 with plant-derived and alum-adjuvanted antigen (0.5 µg/dose). Anti-HBs IgG antibodies in serum, max. ca. 800 mIU/ml	Sojikul et al. (2003)
Nicotiana benthamiana	80 ng/mg TSP (transient expression) 542 ng/mg TSP (transgenic plants)	Transgenic plants and transient expression using transformation-type vectors. Extraction, column concentration, sucrose gradient ultracentrifugation. Ca. 3.5 ng VLPs/µl of final preparation	Mice immunized i.p. 3 ×per 2 w. with plant-derived and alum-adjuvanted antigen (1 µg/dose) Anti-HBs IgG antibodies in serum, max. ca. 400 mIU/ml	Huang and Mason (2004); Huang et al. (2005)

Table 10.1 (continued)

Plant host	Maximum antigen level	Expression system[a], plant material processing	Immunization studies—subject, schedule, and results	References
Nicotiana benthamiana	295 µg/g FW	Transient expression via MagnICON system. Extraction, sucrose gradient ultracentrifugation, immunoaffinity chromatography. VLPs observed in TEM	Mice immunized i.p. 3 × per 2 w. with plant-derived and alum-adjuvanted antigen (1 µg/dose) and boosted after 25 w. with rHBsAg (0.5 µg/dose) Anti-HBs IgG antibodies in serum, max. 400 mIU/ml after priming and 800 mIU/ml after boosting	Huang et al. (2008)

CTB cholera toxin subunit B, *LT-B* heat labile enterotoxin B, *DW* dry weight, *FW* fresh weight, *i.m.* intramuscular injection, *i.p.* intraperiteoneal injection, *mo.* months, *w.* weeks, *rHBsAg* yeast-recombinant purified and alum-adjuvanted HBsAg, *TEM* transmission electron microscope, *TSP* total soluble protein [a]See text for more details

Table 10.2 Progress of prototype plant-based vaccines based on M-HBsAg

Plant host	Maximum antigen level	Expression system[a], plant material processing	Immunization studies—subject, schedule, and results	References
Plant expression only				
Potato	10 ng/mg TSP	Transgenic plants	–	Ehsani et al. (1997)
Plant tissue processing				
Tomato	73 ng/mg TSP	Transgenic plants. Fruit lyophilization. Up to 73 ng of total M-HBsAg/mg TSP	–	Salyaev et al. (2007), (2010)
Lettuce, tobacco	17 µg/g FW	Transgenic plants. Leaf lyophilization Up to 14.6 µg VLPs or 9 µg preS2 per g in fresh preparation	–	Pniewski et al. (2012)
Combined immunisation via injection and edible vaccines				
Potato	0.01 % TSP	Transgenic plants	Mice orally primed by 3 × feeding per 3 w. with raw tubers containing ca. 7.5 µg M-HBsAg with 10 µg CTB/dose and boosted i.p. with rHBsAg (0.5 µg) after 5 w. Anti-HBs IgG in serum, max. 700 mIU/ml. Anti-preS2 antibodies not assayed	Joung et al. (2004)
Potato	0.01 % TSP	Transgenic plants	Mice orally primed by 3 × feeding per 3 w. with raw tubers or extract containing 5 µg M-HBsAg with 10 µg CTB/dose and 2 × boosted (at 8, 32 w.) i.p. with rHBsAg (0.5 µg) and M-HBsAg (0.5 µg). Anti-HBs IgG, max. 600 mIU/ml, and anti-preS2 in serum. Anti-HBs and anti-preS2 S-IgA in feces	Youm et al. (2007)

Table 10.2 (continued)

Plant host	Maximum antigen level	Expression system[a], plant material processing	Immunization studies—subject, schedule, and results	References
Immunization using purified antigen				
Nicotiana benthamiana	40 ng/mg TSP (transient expression) 394 ng/mg TSP (transgenic plants)	Transgenic plants and transient expression using transformation-type vectors. Extraction, column concentration, sucrose gradient ultracentrifugation. Ca. 0.7 ng VLPs/µl of final preparation	Mice immunized i.p. 3 × per 2 w. with plant-derived and alum-adjuvanted antigen (1 µg/dose) Anti-HBs IgG antibodies in serum, max. ca. 1250 mIU/ml	Huang and Mason (2004); Huang et al. (2005)

CTB cholera toxin subunit B, *DW* dry weight, *FW* fresh weight, *i.p.* intraperiteoneal injection, *w.* weeks, *rHBsAg* yeast-recombinant purified and alum-adjuvanted HBsAg, *TSP* total soluble protein [a]See text for more details

Table 10.3 Progress of prototype plant-based vaccines based on L-HBsAg

Plant host	Maximum antigen level	Expression system[a], plant material processing	Immunization studies—subject, schedule, and results	References
Plant expression only				
Tomato	400 ng/g FW	Transgenic plants	–	Lou et al. (2007)
Plant tissue processing				
Lettuce, tobacco	16 µg/g FW	Transgenic plants. Leaf lyophilization Up to 14.6 µg VLPs or 9 µg preS2 per g in fresh preparation	–	Pniewski et al. (2012)
Immunization using purified antigen				
Rice	31.5 ng/g DW chimeric antigen preS1-S	Transgenic plants. Extraction, Sepharose partial purification, CsCl ultracentrifugation. VLPs observed in TEM	Mice immunized i.p. 3 × with 2 w. intervals, using plant-derived antigen (0.5 µg/dose) with Freund's adjuvant Anti-HBs and anti-preS1 in serum, titer 2.1 times higher than control	Qian et al. (2008)

DW dry weight, *FW* fresh weight, *i.p.* intraperiteoneal injection, *w.* weeks, *TEM* transmission electron microscope [a]See text for more details

Salyaev et al. 2007, 2010; Pniewski et al. 2012; Joung et al. 2004; Youm et al. 2007; Lou et al. 2007; Tsuda et al. 1998; Mechtcheriakova et al. 2006; Sainsbury and Lomonossoff 2008; Huang et al. 2009, 2006) played the main role as expression hosts, but other plant species (Kapusta et al. 1999; Chen et al. 2002; Peng et al. 2002; Liu et al. 2005; Sunil Kumar et al. 2003a; Kapusta et al. 2001; Pniewski et al. 2011; Hayden et al. 2012a; Hayden et al. 2012b; Pniewski et al. 2012; Sunil Kumar et al. 2005a; Pniewski 2012; Qian et al. 2008; Mechtcheriakova et al. 2006) and cell or tissue cultures (Sunil Kumar et al. 2006a; Pniewski et al. 2006; Imani et al. 2002; Sunil Kumar et al. 2003a; Sunil Kumar et al. 2003b; Sunil Kumar et al. 2005b; Ganapathi et al. 2007; Mason et al. 2003; Smith et al. 2002; Sojikul et al. 2003) were also utilized. Vectors for *Agrobacterium*-mediated plant transformation mostly conferred marker genes which determined resistance to antibiotics, first of all *nptII*—kanamycin, but also *hpt*—hygromycin in some cases (Imani et al. 2002; Gao et al. 2003; Qian et al. 2008), as well as plants containing the *bar* gene and resistant to herbicide glufosinate (Kostrzak et al. 2009; Pniewski et al. 2011; Hayden et al. 2012a; Hayden et al. 2012b; Pniewski et al. 2012) were also obtained.

Table 10.4 Progress of prototype plant-based vaccines based on HBcAg

Plant host	Max. antigen level	Expression system[a], plant material processing	Immunization studies—subject, schedule and results	References
Antigen purification				
Tobacco	24 µg/g FW	Transgenic plants. Extraction, sucrose gradient centrifugation. Final antigen titer 2^{11}. CLPs observed in TEM	Antigen successfully used for diagnosis of HBV carriers	Tsuda et al. (1998)
Nicotiana benthamiana, cowpea	50–100 µg/g FW 10 µg/g FW	Transient expression via PVX- and CPMV-derived vectors, agroinoculation Extraction, ultracentrifugation. CLPs observed in TEM	–	Mechtcheriakova et al. (2006)
Nicotiana benthamiana	1 mg/g FW	Transient expression via CPMV-derived vectors, agroinoculation CLPs in crude extract observed in TEM	–	Sainsbury and Lomonossoff (2008)
Nicotiana benthamiana	0.8 mg/g FW	Transient expression via BeYDV-derived vectors, agroinoculation Extraction, column concentration, sucrose gradient ultracentrifugation. CLPs observed in TEM	–	Huang et al. (2009)

Table 10.4 (continued)

Plant host	Max. antigen level	Expression system[a], plant material processing	Immunization studies—subject, schedule and results	References
Immunization using purified antigen				
Nicotiana benthamiana	2.4 mg/g FW	Transient expression via MagnICON system. Extraction, column concentration, sucrose gradient ultracentrifugation. CLPs observed in TEM	Mice primed i.p. 2 × 2 w. interval (20 µg/dose), Anti-HBc in serum, mean titer 100,000 Mice primed p.o. 2 × with 2 w. interval (500 µg/dose) and boosted i.n. (100 µg/dose), using plant-derived antigen Anti-HBc in serum, mean titer ca. 15 post priming, 1000 post boosting, 4–5 times higher than i.n. control	Huang et al. (2006)

BeYDV Bean yellow dwarf virus, CPMV Cowpea mosaic virus, PVX Potato virus X, FW fresh weight, *i.n.* intranasal immunization, *i.p.* intraperiteoneal injection, *w.* weeks, *TEM* transmission electron microscope [a]See text for more details

Expression of HBs antigens in transgenic plants was controlled by constitutive promoters—a regular *Cauliflower mosaic virus* (CaMV) 35S promoter (Mason et al. 1992; Kapusta et al. 1999; Rukavtsova et al. 2003; Ehsani et al. 1997; Pniewski et al. 2006; Kostrzak et al. 2009; Pniewski et al. 2011; Gao et al. 2003; Salyaev et al. 2007; Salyaev et al. 2010; Pniewski et al. 2012; Joung et al. 2004; Youm et al. 2007), the 35S derivative or the promoter with a dual enhancer (Lam and Arntzen 1996; Mason et al. 1992; Rukavtsova et al. 2003; Shulga et al. 2004; Richter et al. 2000; Kong et al. 2001; Mason et al. 2003; Thanavala et al. 2005; Dogan et al. 2000; Sojikul et al. 2003; Huang and Mason 2004; Huang et al. 2005; Joung et al. 2004; Youm et al. 2007; Qian et al. 2008), sometimes ubiquitin (Sunil Kumar et al. 2006b; Srinivas et al. 2008; Sunil Kumar et al. 2003a; Sunil Kumar et al. 2003b; Sunil Kumar et al. 2005b; Ganapathi et al. 2007; Hayden et al. 2012a; Hayden et al. 2012b; Sunil Kumar et al. 2005a) or a hybrid promoter octopine synthase–mannopine synthase (OCS–MAS) (Smith et al. 2002). Yet, promoters active in specific organs, such as globulin or glutelin characteristic of grain (Hayden et al. 2012a; Hayden et al. 2012b; Qian et al. 2008), tuber-specific patatin (Shulga et al.

2004; Richter et al. 2000; Joung et al. 2004; Youm et al. 2007), fruit-specific ethylene-forming enzyme (EFE) or 2A11 (Sunil Kumar et al. 2006b; Srinivas et al. 2008; Sunil Kumar et al. 2006a; Sunil Kumar et al. 2003a; Sunil Kumar et al. 2003b; Sunil Kumar et al. 2005b; Sunil Kumar et al. 2005a; Lou et al. 2007), as well as the auxin-inducible MAS promoter (Imani et al. 2002), were tried as well. As polyadenylation signals, *Agrobacterium* terminators such as OCS or especially NOS were commonly utilized, but the signals of plant origin were also used (Richter et al. 2000; Kong et al. 2001; Mason et al. 2003; Thanavala et al. 2005; Hayden et al. 2012a; Hayden et al. 2012b; Dogan et al. 2000; Smith et al. 2000; Sojikul et al. 2003; Huang and Mason 2004; Huang et al. 2005). There were many attempts to intensify gene expression and consequently accumulation of a particular HBV antigen. Among those, the following can be mentioned: (1) optimization of the coding sequence according to a plant pattern of codon usage (Mason et al. 2003; Sojikul et al. 2003; Lou et al. 2007), (2) transcription activation by 5′-untranslated region (UTR) sequences coming from plant viruses such as tobacco etch virus (TEV) or *Alfalfa mosaic virus* (AlMV) (Mason et al. 1992; Richter et al. 2000; Kong et al. 2001; Mason et al. 2003; Thanavala et al. 2005; Dogan et al. 2000; Sojikul et al. 2003; Huang and Mason 2004; Huang et al. 2005; Joung et al. 2004; Youm et al. 2007), and (3) enhanced stability of a synthesized antigen by its deposition in defined compartments following conjugation with targeting sequences, such as the ER-retention signal (Sunil Kumar et al. 2006b; Srinivas et al. 2008; Sunil Kumar et al. 2006a; Sunil Kumar et al. 2003a; Sunil Kumar et al. 2003b; Sunil Kumar et al. 2005b; Ganapathi et al. 2007; Richter et al. 2000; Mason et al. 2003; Hayden et al. 2012a; Hayden et al. 2012b; Sunil Kumar et al. 2005a; Sojikul et al. 2003; Salyaev et al. 2010; Lou et al. 2007) or storage protein signals (Richter et al. 2000; Mason et al. 2003; Hayden et al. 2012a; Hayden et al. 2012b; Sojikul et al. 2003; Huang and Mason 2004; Huang et al. 2005).

Nevertheless, production of HBsAg was rather not orchestrated with a host or an expression cassette. S-HBsAg content usually ranged from 0.01 to several micrograms per gram of fresh weight (FW) or equivalently tens of a few hundreds of nanograms/milligrams of total soluble protein (TSP) (Lam and Arntzen 1996; Mason et al. 1992; Kapusta et al. 1999; Rukavtsova et al. 2003; Sunil Kumar et al. 2006b; Ehsani et al. 1997; Shulga et al. 2004; Zhao et al. 2000; Srinivas et al. 2008; Chen et al. 2002; Peng et al. 2002; Liu et al. 2005; Sunil Kumar et al. 2006a; Pniewski et al. 2006; Imani et al. 2002; Ganapathi et al. 2007; Kapusta et al. 2001; Kong et al. 2001; Gao et al. 2003; Dogan et al. 2000; Sunil Kumar et al. 2005a; Sojikul et al. 2003; Joung et al. 2004; Youm et al. 2007), rarely ≥10 μg/g FW (Sunil Kumar et al. 2003a; Sunil Kumar et al. 2003b; Sunil Kumar et al. 2005b; Kostrzak et al. 2009; Pniewski et al. 2011; Richter et al. 2000; Mason et al. 2003; Thanavala et al. 2005; Hayden et al. 2012a; Hayden et al. 2012b; Pniewski et al. 2012; Smith et al. 2000; Sojikul et al. 2003; Huang et al. 2005). In the case of M- and especially L-HBsAg, those values were even lower, probably due to the restricted ability to form stable VLPs, and ranged from 10 to 100 ng/mg TSP or 0.4 to 2 μg/g FW (Ehsani et al. 1997; Huang and Mason 2004; Huang et al. 2005; Salyaev et al. 2007; Salyaev et al. 2010; Joung et al. 2004; Youm et al. 2007; Lou et al. 2007). An interesting approach

to improve expression of the crucial epitope preS1 of L-HBsAg was provided by its fusion to truncated S-HBsAg as a carrier. A gene-encoding chimeric protein was placed under the control of the glutelin promoter and expressed in rice seeds (Qian et al. 2008). Even though the fusion antigen assembled into VLPs, its total yield was low—only ca. 30 ng/g dry weight (DW).

It may be supposed that different processes affected HBsAg expression, although respective detailed studies were not conducted. Apart from the position effect or gene silencing commonly observed in transgenic plants, some more specific phenomena might have occurred. For instance, cell ultrastructure or functioning might have been somehow disrupted by synthesized HBsAg, due to its affinity to membranes. Perhaps, apart from expression cassette elements, a positive effect on HBsAg accumulation could be found for the general metabolism type and cell substructure. The capacity of plant cells to supply membrane lipids for HBsAg assembly and later to deposit or secrete VLPs may play an essential role, as in other cells (Michel and Tiollais 2010). It could be observed that still not very high, but relatively larger HBsAg contents were observed in suspension cultures, where the antigen could be secreted (Sunil Kumar et al. 2003a, 2003b, 2005b; Smith et al. 2002), or in loose and wide mesophyll or parenchymatic cells of tubers or grains (Kostrzak et al. 2009; Pniewski et al. 2011; Richter et al. 2000; Kong et al. 2001; Mason et al. 2003; Thanavala et al. 2005; Hayden et al. 2012a, b; Huang et al. 2005; Pniewski et al. 2012; Tsuda et al. 1998) in comparison to the "closely packed" cells of tobacco seeds (Sunil Kumar et al. 2006b). Yet, promising recent reports indicate that transgenic plants can produce reasonable amounts of HBsAg. Lettuce containing an unmodified coding sequence controlled by the regular 35S promoter expressed S-HBsAg in leaves at a mean level of 20 μg/g FW and maximum 60 μg/g FW (Pniewski et al. 2011), while M- and L-HBsAg expressed at 16–17 μg/g FW (Pniewski et al. 2012). In another study, S-HBsAg was accumulated in maize seeds up to 71 μg/g (Hayden et al. 2012a; Hayden et al. 2012b), when the antigen sequence was conjugated with a signal peptide of storage protein and the whole coding sequence placed under the control of the multiplied seed-specific globulin promoter. However, even these values remain approximately four to six times lower than the best results obtained for transient expression systems, when antigens can be expressed in some measure outside plant control mechanisms.

For the past few years, the role of transient expression methods in the production of HBV antigens has evidently increased. Although initially S- and M-HBsAg were expressed at a rather low level, 60–490 ng/g DW in tomato (Srinivas et al. 2008) or tens of nanograms/milligrams TSP (Huang and Mason 2004; Huang et al. 2005) in *N. benthamiana*. Probably this slight efficiency was caused by the applied method—tissue infiltration with ordinary *Agrobacterium* strains carrying vectors typical of stable transformation. Only when one of the MagnICON® system (Gleba et al. 2005) was exploited, the S-HBsAg content reached 295 μg/g FW (Huang et al. 2008). Regrettably, this result still remains unique.

HBcAg stands alone among HBV antigens. Besides its biochemical properties and biological function, studies on plant expression took also a different direction. Although the first report on a relatively efficient HBcAg expression (24 μg/g FW)

in transgenic tobacco was published as early as in 1998 (Tsuda et al. 1998), for years this antigen seemed practically forgotten since efforts have been focused on HBsAg. A potential role of HBcAg as an essential component of therapeutic vaccines or a carrier for various epitopes once more focused attention on this antigen. In recent years, HBcAg has been produced only using transient expression methods, e.g., conventional MagnICON® (Huang et al. 2006) or novel viral vectors based on potato virus X (PVX), *Cowpea mosaic virus* (CPMV) or *Bean yellow dwraf mosaic virus* (BeYDV) (Mechtcheriakova et al. 2006; Sainsbury and Lomonossoff 2008; Huang et al. 2009). HBcAg was usually produced in several times larger quantities when compared to HBsAg, and was as high as 0.5–2 mg/g FW (Mechtcheriakova et al. 2006; Sainsbury and Lomonossoff 2008; Huang et al. 2009, 2006) in *N. benthamiana,* although merely 10 μg/g FW in cowpea (Mechtcheriakova et al. 2006).

Irrespective of production scale, HBV antigens in plant cells preserved their physiochemical properties and antigenicity. Size and density of the antigens were correct, as for those from human plasma or derived from other expression systems (Mason et al. 1992; Ehsani et al. 1997; Shulga et al. 2004; Pniewski et al. 2006, 2011, 2012; Sunil Kumar et al. 2005b, 2005a; Ganapathi et al. 2007; Mason et al. 2003; Hayden et al. 2012b; Dogan et al. 2000; Smith et al. 2002; Sojikul et al. 2003; Huang and Mason 2004; Huang et al. 2005, 2008; Joung et al. 2004; Tsuda et al. 1998; Mechtcheriakova et al. 2006; Sainsbury and Lomonossoff 2008; Huang et al. 2009, 2006). Glycosylation of HBV antigens in plants remains obscure at this moment. According to most reports, this process did not occur, analogously to yeast (Shulga et al. 2004; Pniewski et al. 2006; Sunil Kumar et al. 2005b; Ganapathi et al. 2007; Hayden et al. 2012b; Smith et al. 2002; Huang et al. 2005; Huang et al. 2008; Joung et al. 2004; Mechtcheriakova et al. 2006; Huang et al. 2009; Huang et al. 2006), but HBsAg proteins of a slightly higher mass, putatively due to glycosylation, were also observed (Pniewski et al. 2011; Pniewski et al. 2012). Yet, it may be stated that in any case, plant-produced HBV antigens retained their native structure, as confirmed by enzyme-linked immunosorbent assay (ELISA) or similar tests using specific antibodies or diagnostic kits (Lam and Arntzen 1996; Mason et al. 1992; Thanavala et al. 1995; Kapusta et al. 1999; Rukavtsova et al. 2003; Sunil Kumar et al. 2006b, a, 2003a, b, 2005b, a; Ehsani et al. 1997; Shulga et al. 2004; Zhao et al. 2000; Srinivas et al. 2008; Chen et al. 2002; Peng et al. 2002; Liu et al. 2005; Pniewski et al. 2006, 2011, 2012; Imani et al. 2002; Ganapathi et al. 2007; Kapusta et al. 2001; Kostrzak et al. 2009; ; Richter et al. 2000; Kong et al. 2001; Mason et al. 2003; Gao et al. 2003; Thanavala et al. 2005; Hayden et al. 2012a, b; Pniewski 2012; Dogan et al. 2000; Smith et al. 2002; Sojikul et al. 2003; Huang and Mason 2004; Huang et al. 2005, 2008; Salyaev et al. 2007, 2010; Joung et al. 2004; Youm et al. 2007; Lou et al. 2007; Qian et al. 2008; Tsuda et al. 1998; Mechtcheriakova et al. 2006; Sainsbury and Lomonossoff 2008; Huang et al. 2009, 2006). Consequently, HBV antigens formed VLPs or CLPs, which were observed directly in plant cells or purified (Mason et al. 1992; Pniewski et al. 2011; Kong et al. 2001; Smith et al. 2002; Huang et al. 2008; Lou et al. 2007; Qian et al. 2008; Tsuda et al. 1998; Mechtcheriakova et al. 2006; Sainsbury and Lomonossoff 2008; Huang et al. 2009, 2006).

Summing up, numerous experiments demonstrated that plants can be effective bioreactors for the production of native HBV antigens. Plants bearing antigens have been then exploited for oral immunization trials, directly as edible vaccines or after some processing as plant-derived formulations. Both types of oral vaccines were also used in combined, injection–oral immunization. A distinct issue is connected with the purification of the antigens for subsequent injection vaccination. Those research stages are presented in Tables 10.1, 10.2, 10.3 and 10.4 and discussed below.

Oral Immunization Using Edible Vaccines and Plant-Derived Formulations

At the turn of the 1990s and 2000s, the terms "plant-based vaccines" and "edible vaccines" were used synonymously. Positive results of the first oral immunization trials using plants bearing LT-B, CTB, or S-HBsAg raised genuine enthusiasm, thus the oral route of vaccine delivery has been investigated in many trials. Potential problems connected with edible vaccines, such as their perishable and bulky form or forced ingestion, seemed to wane, since effectiveness of vaccination seemed to be a priority. Plant-based prototype vaccines against HBV have been investigated according to two main methodological approaches.

Chronologically, the first one was founded on the exclusively oral administration of plant material, without any injection or exogenous adjuvant (Kapusta et al. 1999). The idea of such an immunization method was to provide a potential vaccine as simply as possible. The "opening" experiment, conducted with the use of raw lettuce leaves or lupin callus tissue containing S-HBsAg, proved that it was possible to evoke anti-HBs antibodies in serum above the protective minimum, i.e., 10 mIU/ml. Moreover, it appeared that relatively low doses of unadjuvanted S-HBsAg, ca. 0.5–1 μg, as well as longer intervals between priming and boosting (1 or 2 months) were adequate for immunization. The importance of a proper regime of antigen delivery was proven in that and subsequent experiments. When mice were fed once over the course of the experiment, they responded better than those fed in multiple doses (Kapusta et al. 1999). In turn, when volunteers consumed lettuce at days 0, 7, and 30 instead of 0 and 30 or later, the antibody titer did not reach the protective minimum (Kapusta et al. 2001). However, it should be admitted that although in both experiments indisputable success was achieved such as induction of systemic humoral response via antigen delivery to intestinal mucosa, the titer of anti-HBs antibodies only fluctuated around the minimal protection level.

Oral immunization with the use of edible vaccines appeared to be insufficient, not to mention their onerous application mode. Besides, vaccination by consumption might be conducive to oral tolerance acquisition. According to contemporary knowledge on functioning of the gut-associated lymphoid tissue (GALT), a "fed" antigen, especially when abundantly loaded, could be split into lower sub-doses, frequently or for an extended period exposed to GALT, then recognized as neutral or even a dietary component and thus tolerated (Swarbrick et al. 1979; Peng et al. 1989; Friedman and

Weiner 1994; Strobel 2001; Mowat 2003). Therefore, a new form of a vaccine was required to immunize by a controlled regime and convenient delivery in "one shot." Lyophilized tissue could meet these conditions due to its size-reduced and durable form and a defined antigen dose. Processed plant tissue containing only 100 ng of unadjuvanted S-HBsAg within 1 or 2 months evoked systemic response above the anti-HBs protective titer (Pniewski et al. 2011), while high doses were ineffective. Still, evoked responses were too low (maximum 20 mIU/ml) for practical use.

Recurring difficulties with oral immunization using plant-based vaccines against HBV and other blood-borne pathogens gradually caused a revision of views on the effectiveness of plant-based vaccines and the role of oral tolerance (Mestecky et al. 2007; Wang and Coppel 2008). Furthermore, it was experimentally confirmed that orally delivered plant-associated S-HBsAg induced growth of a subpopulation of regulatory T (Treg) lymphocytes (Kostrzak et al. 2009), suppressors of the active systemic response, and mediators of oral and generally mucosal tolerance development (Taams et al. 1998). That posed an obvious obstruction to oral plant-based vaccines, since it seemed that natural plant components induced mucosal tolerance, which then might expand to the associated antigen. Although the intensity of the probable oral tolerance correlated with the antigen dosage, even extremely low antigen doses (several nanograms) stimulated a certain growth of the Treg population. It is also possible that S-HBsAg, coming from a non-mucosal pathogen, was recognized by the GALT as a neutral antigen (Matzinger 1994). Moreover, simultaneous to the suppression of systemic response, the dose-dependent production of anti-HBs S-IgAs was observed (Kostrzak et al. 2009; Pniewski et al. 2011; Hayden et al. 2012a; Youm et al. 2007). This process may be considered as disadvantageous, especially when correlated with declined systemic response, which is required in the case of blood-borne pathogens, such as HBV. Overall, specific mechanisms of mucosal immune response, therein S-IgA production and/or oral tolerance, most probably constitute a barrier to vaccine antigens, even such strong immunogens as HBsAg, as long as they come from non-mucosal pathogens.

Alongside HBsAg, some problems with oral immunization could be observed also for HBcAg, which is a naturally strong antigen (Böcher et al. 2001). The partially purified, high-dosed (500 μg) and alum-adjuvanted antigen induced no more than a mild systemic response when orally delivered, while an intensive reaction was elicited by intranasal immunization (Huang et al. 2006). Although nasal and intestinal mucosas comprise the mucosa-associated lymphoid tissue (MALT), the nasal cavity and the gut lumen are different milieus, in which distinct mechanisms of mucosal response are active. On the other hand, successful intranasal immunization using HBcAg may suggest that besides the intestinal membrane, other mucosal membranes could be appropriate recipients for anti-HBV vaccines.

Edible vaccines in combined injection-oral immunization

According to the second approach, edible vaccines bearing HBV antigens, S-, or M-HBsAg were used for effective immunization. However, it has to be emphasized

that oral antigen delivery was only a part of the whole immunization schedule. Typically, intramuscular or intraperitoneal injection of HBsAg preceded the administration of edible vaccines or, more rarely, this sequence was inversed. What is more, edible vaccines were administered by a reverse regime than the one presented above. The antigens were high dosed and repeatedly delivered, as well as usually supplemented with a strong mucosal adjuvant-activating GALT, such as CTB or LT-B (Richter et al. 2000; Kong et al. 2001; Mason et al. 2003; Hayden et al. 2012a; Hayden et al. 2012b; Joung et al. 2004; Youm et al. 2007). Still, consecutive (every few days) consumption of raw plant tissue, mainly potato tubers (Richter et al. 2000; Kong et al. 2001; Mason et al. 2003; Joung et al. 2004; Youm et al. 2007) bearing adjuvanted HBsAg and dosed as plentifully as from several up to even 42 μg/mouse (Kong et al. 2001) or several hundreds μg/human (Thanavala et al. 2005), elicited a significant anti-HBs systemic response, maximum 700–5,000 mIU/ml, comparably to standard vaccination (Richter et al. 2000; Kong et al. 2001; Mason et al. 2003; Gao et al. 2003; Thanavala et al. 2005; Joung et al. 2004; Youm et al. 2007). Thus, although the immunization procedure was not strictly oral, it was effective and for that reason, this approach to edible vaccines was broadly accepted. Apart from the effectiveness of the injection-oral immunization, some other essential findings might be noticed. A single antigen injection appeared to be sufficient for effectual oral boosting (Richter et al. 2000; Kong et al. 2001; Mason et al. 2003; Youm et al. 2007). This suggests that some immune alertness is crucial for the efficacy of oral booster vaccination. That assumption can be also corroborated by results of experiments when volunteers exhibiting some anti-HBs antibodies, maintained for years after a regular 3-dose rHBsAg injection vaccination, responded to the antigen in the edible vaccine (Thanavala et al. 2005). On the other hand, when mice were fed multiply with plant tissue containing S-HBsAg, they were not sensitized via that way to the antigen and did not react to afterward injected rHBsAg (Gao et al. 2003).

However, although effective when applied according to the combined immunization procedure, edible vaccines still would be very difficult to be regularly employed. Problems with administration of perishable and bulky plant tissue, sometimes containing harmful secondary metabolites as in tubers, remained unsolved and decisive. The usage of CTB or LT-B as adjuvants, notwithstanding their declared harmlessness, could also raise controversies in the case of mass-scale vaccination (Williamson et al. 1999). Yet, non-adjuvanted edible vaccines induced only low or no reaction, even when it followed injection priming (Kong et al. 2001; Gao et al. 2003). Plant-based vaccines against HBV, whether edible or derived formulations but solely orally delivered, seemed to be a part of the vicious circle.

A Potential Solution of Oral Immunization Barriers: Confluence of Combined Vaccination Pattern and Plant-Derived Oral Formulations

All in all, lingering problems revealed that a really efficacious and practical-in-use plant-based oral vaccine against HBV appeared much more difficult to develop than it had been initially assumed. Two quintessential problems—inappropriate vaccine

delivery and its form—seemed to exclude each other. As a consequence, interest in plant-based anti-HBV oral vaccines has been lessening. On the other hand, knowledge gained in previous projects may be invaluable if right tracks among collected data would be recognized.

A possible solution is, in fact simple, coupling both previous methodological approaches. Oral immunization against HBV could be based on a combined, injection-oral antigen delivery, but the oral component would be a plant-derived, processed formulation. Priming via a parenterally delivered antigen apparently somehow reduces the risk of oral tolerance by making GALT susceptible to the oral booster (Richter et al. 2000; Kong et al. 2001; Mason et al. 2003; Gao et al. 2003; Thanavala et al. 2005; Youm et al. 2007). This in turn can be performed using a stable and easy-to-handle plant-derived formulation with a concentrated and defined HBsAg dose (Pniewski et al. 2011; Hayden et al. 2012a; Hayden et al. 2012b; Pniewski 2012; Pniewski et al. 2012). In this way, both the main above-mentioned pitfalls of oral plant-based vaccines would be overcome.

Recent reports may serve as primary confirmation to the proposed approach. In both, mice were intramuscularly primed with a commercial recombinant vaccine, then orally boosted using S-HBsAg contained within pellets obtained from grain pulp after coarse oil extraction using organic solvents (Hayden et al. 2012a; Hayden et al. 2012b) or lyophilized leaves (Pniewski 2012). In the first experiment, the high-dosed (>100 μg) S-HBsAg, adjuvanted with LT-B and three times administered after 15 weeks post injection, elicited up to 4,600 mIU/ml of anti-HBs antibodies (Hayden et al. 2012a). The second experiment went slightly further, as the low-dosed antigen (maximum 200 ng) and without an exogenous adjuvant was orally delivered 6 and 12 weeks post injection. However, that regime appeared to be also sufficient, as it induced around 800 mIU/ml of anti-HBs, comparable to three doses of the injected antigen (Pniewski 2012). Regardless of the promising results, plant-derived oral formulations would require a major upgrade. Mainly, stability of HBsAg during plant material processing and storage of preparations needs to be comprehensively improved. In the case of S-HBsAg, even 90% of VLPs were destroyed during freeze-drying, although the antigen was later maintained when stored (Pniewski et al. 2011). For M- and L-HBsAg, especially preS domains, and VLPs were susceptible to alteration or degradation (Pniewski et al. 2012). In turn, oil extraction and pulp preparation did not significantly affect S-HBsAg, but after 1 week of storage, 25–45% of S-HBsAg were lost (Hayden et al. 2012a; Hayden et al. 2012b). Yet, if plant-derived oral formulations are optimized, there should not be obstacles to their use as periodically administered anti-HBV booster vaccines. Moreover, the injection component for priming can also be of plant origin.

Plant-Derived Injection Vaccines

For years, the term “plant-based vaccines” had been associated rather with oral ones. However, S-HBsAg expressed for the first time in plants (Mason et al. 1992)

was just purified and administered by injection (Thanavala et al. 1995). In fact, this research field developed in parallel, and recently essential progress has been noted. Although processing of such vaccines is surely more complicated and costly than oral ones, parenteral delivery of HBV antigens had a pivotal superiority, since appropriate efficacious protocols had already been well developed.

Delivery via injection requires a purified antigen, which in turn involves the highest possible scale of the antigen production to make the whole process worthwhile. In the first experiments HBV antigens for purification, not only S-HBsAg but also M-HBsAg, the chimeric antigen preS1-S (Qian et al. 2008) and HBcAg, were produced in transgenic plants or transiently, but via regular *Agrobacterium* vectors. Thus, yields were relatively low, from ca. 10 ng to several microgram/gram FW or equivalently tens or hundreds of nanogram/microgram TSP (Mason et al. 1992; Dogan et al. 2000; Sunil Kumar et al. 2005a; Sojikul et al. 2003; Huang and Mason 2004; Huang et al. 2005; Qian et al. 2008), and only exceptionally >20 μg/g FW in the case of HBcAg (Tsuda et al. 1998). Although antigens were sufficiently purified for injection (Thanavala et al. 1995; Sojikul et al. 2003; Huang and Mason 2004; Huang et al. 2005; Qian et al. 2008), the yields still remained inadequate. When soybean suspension culture was adopted (Smith et al. 2002), where S-HBsAg could be secreted, the yield increased considerably (74 μg/g FW). However, a real breakthrough came when the MagnICON® or virus-based transient expression systems were employed. Only then were S-HBsAg (Huang et al. 2008) and HBcAg (Mechtcheriakova et al. 2006; Sainsbury and Lomonossoff 2008; Huang et al. 2009; Huang et al. 2006) produced with robust yields, as high as 0.3–2 mg/g FW. Assembly of HBV antigens into VLPs or CLPs made it possible to exploit the standard (McAleer et al. 1984; Han et al. 2006) and a relatively inexpensive technique of purification of macromolecular complexes, such as ultracentrifugation, usually in sucrose (Mason et al. 1992; Thanavala et al. 1995; Dogan et al. 2000; Smith et al. 2002; Sojikul et al. 2003; Huang and Mason 2004; Huang et al. 2005; Huang et al. 2008; Tsuda et al. 1998; Huang et al. 2009; Huang et al. 2006) or cesium chloride gradient (Mason et al. 1992; Sunil Kumar et al. 2005a; Qian et al. 2008). These methods were used as only one processing step or as preceding more advanced techniques such as ultrafiltration (Thanavala et al. 1995; Sojikul et al. 2003; Huang and Mason 2004; Huang et al. 2005; Huang et al. 2009; Huang et al. 2006) or immunoaffinity purification (Mason et al. 1992; Huang et al. 2008). The total efficiency or antigen concentration after the production phase and downstream processing amounted to 0.4–3.5 μg/ml (Dogan et al. 2000; Sunil Kumar et al. 2005a; Smith et al. 2002; Sojikul et al. 2003; Huang and Mason 2004; Huang et al. 2005) and still could be increased up to even 100 μg/ml (Thanavala et al. 1995), which is the valid value for commercial vaccines.

However, the most significant aspect was that plant-derived VLPs or CLPs had identical properties and evoked comparable systemic humoral responses as commercial vaccines (Thanavala et al. 1995; Sojikul et al. 2003; Huang and Mason 2004; Huang et al. 2005; Huang et al. 2008; Joung et al. 2004; Youm et al. 2007; Qian et al. 2008; Huang et al. 2006). Injected S-HBsAg elicited anti-HBs response of up to 800 mIU/ml (Thanavala et al. 1995; Sojikul et al. 2003; Huang and Mason 2004;

Huang et al. 2005; Huang et al. 2008; Joung et al. 2004), M-HBsAg—1,165 mIU/ml (Huang et al. 2005), and also induced anti-preS2 antibodies (Youm et al. 2007), while chimeric preS1-S induced anti-preS1 antibodies at a titer twofold higher than in the control (Qian et al. 2008). In turn, plant-derived HBcAg evoked the same antibody titer as the antigen recombined in *E. coli* (Huang et al. 2006). These results showed evidently that plant-derived HBV antigens used as injection vaccines are equivalent to classical ones.

Prospective View

Research on plant-based vaccines against HepB has been conducted for more than 20 years. In spite of many efforts and more or less optimistic prognoses (Streatfield 2005; Sunil Kumar et al. 2007; Daniell et al. 2009), none is commercially produced or tested in an authentic clinical trial. What is worse, concerns might arise about the soundness of further studies on those, not to mention their possible introduction. In the meantime though, thanks to vaccines produced in yeast or mammalian cells, as well as some progress in therapy, prophylaxis and control of HepB have rallied to a large extent (Kew 2010; Romano et al. 2011; Michel and Tiollais 2010; Mallet et al. 2011) (see above).

However, that improvement mostly refers to highly and relatively developed countries. Large areas of Asia and Africa are still regions of high HBV prevalence (see above) and insufficiently covered by easily accessible HepB vaccines. At the same time, although the price of the vaccines considerably dropped (US$ 1–10, up to ca. US$ 60 in developed countries), problems with their distribution (e.g., the cold chain), deficient infrastructure and education, as well as a certain shortage of highly qualified healthcare workers result in ineffective immunization or delayed programs of HepB prevention (Kew 2010; WHO Media Centre 2012; Romano et al. 2011). Apart from problems with prophylaxis, HepB therapy remains an economical barrier for inhabitants of poorer countries, the most threatened by HBV and HCC (Yuen and Lai 2011). Taking all those facts into consideration, easily implementable vaccines and methods of anti-HepB prevention continue to be necessary, hence there is still field for plant-based vaccine application.

For years, however, the original concept of plant-based vaccines against HepB has undergone a profound metamorphosis. The catchphrase “edible vaccines” has become archaic, since they turned out to be completely unfeasible. Entirely oral immunization also seems problematic. However, a major part of assumptions for plant-based vaccines is still topic. Hence, they may be considered as alternative or auxiliary to standard ones (Fig. 10.1). Last reports undeniably showed that plant-derived injections or oral formulations are effective (Hayden et al. 2012a; Hayden et al. 2012b; Pniewski 2012; Huang et al. 2008; Huang et al. 2006), together with potential for cost-effective production and processing (for review, Komarova et al. 2010; Tiwari et al. 2011; Tiwari and Vyas 2011; Kwon et al. 2013; Scotti and Rybicki 2013).

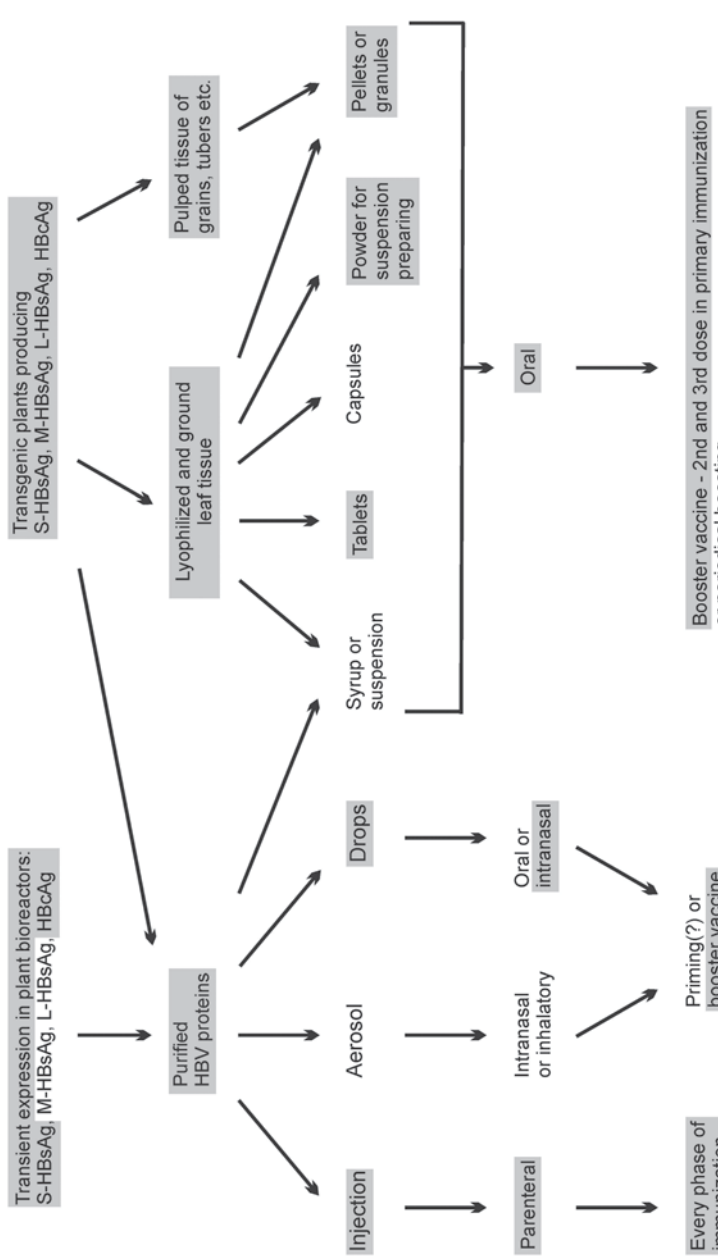

Fig. 10.1 Potential plant-derived vaccines for immunization against Hepatitis B Virus. Note: *gray shading*—stages of technology achieved hitherto, see text for details

From among the two types of potential plant-derived anti-HBV vaccines, a more promising scenario can be written for those delivered by injection. In short, they have prospects as equivalent to or even more beneficial than those obtained in traditional systems. This is not only due to their efficacy comparable to standard vaccines (Thanavala et al. 1995; Sojikul et al. 2003; Huang and Mason 2004; Huang et al. 2005; Huang et al. 2008; Joung et al. 2004; Youm et al. 2007; Qian et al. 2008; Huang et al. 2006) and known vaccination protocols, distribution procedures, etc. Purification methods of HBV antigens have also been developed and need only to be adjusted for plant material. Milligram yields of S-HBsAg or HBcAg provide a solid basis for abundant production (Huang et al. 2008; Sainsbury and Lomonossoff 2008; Huang et al. 2009; Huang et al. 2006). Special facilities adapted to transient expression technology and fulfilling policies of good manufacture practice (GMP) have already been developed (Lai and Chen 2012), thus they might be easily exploited for the manufacture of HBV antigens on a semi- or technical scale. However, some issues need to be explained in the future. The first question is whether plants may be competitive to other expression systems. Nowadays, the yield of transient expression makes up only ca. 5% of yeast production (McAleer et al. 1984), but probably it can still be increased (Tiwari et al. 2011) and after that, it is economically comparable. In turn, it is almost certain that plant systems would be a lot more cost effective than mammalian cells (Tiwari et al. 2011). The second, i.e., nativity of the products may also be positively solved. Apart from the fact that plant-produced HBV antigens are non- or "plant-glycosylated," they retained their antigenicity and immunogenicity. Moreover, plants of an engineered glycosylation pathway were constructed (Bosch and Schots 2010). Hence, plant-derived products would be identical to the original one, for instance "humanized." Other issues still require corroboration or elaboration, e.g., repeatability of S-HBsAg transient high expression or M/L-HBsAg synthesis in that system. When the latter is feasible, a plant-derived vaccine would be not only the second generation but the third generation as well. Regardless of the fact, research on plant-derived anti-HBV injection vaccines, especially based on S-HBsAg and HBcAg, reached a point, where large-scale vaccination trials are technically feasible and may be expected in the near future.

On the other hand, there is no denying that HBV antigens produced via transient systems used as injection vaccines would be in fact generic vaccines. Taking into consideration the present state, they would contain S-HBsAg only. Admittedly, plants can provide HBcAg, but its use for therapeutic vaccines is still under research, while M/L-HBsAg awaits an efficient transient expression at all. Potential plant-derived injection vaccines would involve complex production and processing facilities, and require cold chain distribution and delivery utensils. Consequently, they likely would bring about only slightly lower costs in comparison to the present vaccines.

There are some possibilities which would to some extent make vaccines founded on purified HBV antigens more readily available. For instance, they could be distributed in a durable lyophilized form (Diminsky et al. 2000), suspended directly before injection. Purified HBs antigens might also be used as oral vaccines. Orally

delivered purified HBsAg supplemented with different adjuvants elicited anti-HBs systemic humoral response (Borges et al. 2007; Shukla et al. 2008; Kapusta et al. 2010), and a potential therapeutic vaccine against chronic HepB might also be applied in that way (Safadi et al. 2003). One might presume that purified HBV antigens could be administered not only as oral but generally also as mucosal vaccines, similarly to the already-tried intranasal delivery of plant-derived HBcAg (Huang et al. 2006). However, supposing that the above options were successfully verified, one should be aware that such a mode of anti-HBV vaccine administration would not be a panacea. They would also require additional equipment (applicators, etc.), while one cannot count on the elimination of the cold chain, not to mention further immunization studies. In general, it may be assumed that plant-derived vaccines founded on purified antigens, both parenteral or mucosal, would support prophylaxis against HepB (Fig. 10.1). However, issues of simplified distribution, vaccination, and common access probably would remain, unless they could be solved by socio-economic and political means (reimbursements, public capital investments, etc.).

In that situation, there is still a certain chance for plant-derived oral formulations, which can be considered as yet meeting the original concept of cheap and commonly available vaccines against HepB. However, based on previous studies and especially the latest results, a potential oral vaccine possibly will be considered as a booster one only. This would be used as the second or third dose in primary combined parenteral–mucosal immunization, or as periodically administered thereafter. Plant tissue for the oral vaccine could be lyophilized (Pniewski et al. 2011; Salyaev et al. 2007; Salyaev et al. 2010; Pniewski et al. 2012) or extracted and pulped (Hayden et al. 2012a; Hayden et al. 2012b). Afterward, these semi-products can be converted into tablets (Pniewski et al. 2011), pellets (Hayden et al. 2012a; Hayden et al. 2012b), or others (Fig. 10.1), such as portioned powder for suspension or syrup, or the recently obtained capsules as a vaccine against tuberculosis (Lakshmi et al. 2013). Such formulations, especially tablets or capsules, which exhibit relative stability at ambient temperature, a concise and convenient form, and a standardized antigen dose, would facilitate control of the immunization regime, and consequently—its efficacy. Such oral vaccines also offer an option for the modification of their formulas to be optimal for immunogenicity. Activity of the plant-associated antigen could be augmented by added interleukins and other immunomodulators or various adjuvants, both artificial such as the tested CpG oligos, chitosan, bile salts or other lipids (Borges et al. 2007; Shukla et al. 2008; Wee et al. 2008), or natural plant substances such as oils, terpenoids, saponins, lectins, and many others (Skene and Sutton 2006; Vajdy 2011). Moreover, it can be assumed that oral vaccines could contain a particular HBV antigen, as well as be analogous to the current third-generation vaccines and contain two or more immunogens.

At this moment, any potential anti-HBV oral vaccine needs to be developed or optimized. Especially, improvement of processing yield and antigen preservation in long-term storage are a challenge. Yet, as primary reports show, oral vaccines founded on S-HBsAg are relatively the closest to be fully elaborated (Pniewski et al. 2011; Hayden et al. 2012a; Hayden et al. 2012b). Probably, so do the vaccines based on HBcAg, since that antigen can be highly expressed (Tsuda et al. 1998) and

assemble stable CLPs. Vaccines containing M- and L-HBsAg appear to be more complicated, due to their lower expression potential and susceptibility of those to degradation during tissue processing (see above) (Pniewski et al. 2012). However, those problems do not seem to be unsolvable. For instance, processing yield can be increased by manipulation of physical conditions, optimization of extraction mixtures or addition of lyoprotectants, stabilizers, etc.

Summarizing, although years of efforts on making a plant-based vaccine against HepB brought many disappointments, knowledge and technologies have significantly progressed. Efficient stable and/or transient expression systems for each HBV antigen, methods of plant tissue processing and antigen purification, and new effective immunization procedures provide a solid rationale for further research and possible applications. Purified antigen(s) can be delivered by injection as a regular vaccine or through mucosas. Tablets or capsules prepared from processed tissue can be administered orally as a booster vaccine. Both vaccine types may be combined in the parenteral–mucosal immunization procedure (Fig. 10.1).

Plant-derived injection vaccines as generics would probably have an advantage over oral formulations. It may be expected that the first ones would be much earlier manufactured according to regulations of GMP and approved for clinical use. However, oral anti-HBV vaccines, even optimized, would be a new type of a vaccine. Hence, they would entail thorough tests on their efficacy, biosafety, bioequivalence, etc.

As a final conclusion, it can be stated that truly efficacious and reasonably priced plant-based vaccines against HepB are sound and within reach. Plant-derived injection vaccines can supplement or even replace the present vaccines. Oral formulations which would substitute booster vaccines could still be seen as a worthwhile alternative. On the whole, plant-based vaccines can help to fight persisting HepB in many countries worldwide.

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Chapter 11
Plant-Based Vaccines Against Toxoplasmosis

Marina Clemente

Introduction

Currently, the production of heterologous proteins in plants is an alternative to other expression systems based on microorganisms and animal cells. Plant expression systems combine the ability to make posttranslational modifications with minimum requirements for growth and virtually unlimited biomass production (Karg and Kallio 2009). In addition, since plants are not contaminated with animal pathogens and/or bacterial endotoxins, the proteins produced in plants are safer for use in human and veterinary medicine (Desai et al. 2010). On the other hand, plant systems are versatile in terms of expression platforms and production scale, and thus may adapt quickly to the market demand (Paul and Ma 2011).

In addition to their use as bioreactors, plants can be used as potential delivery systems for oral vaccines (Pelosi et al. 2012). In particular, plant tissues provide protection and prevent degradation of the antigen when it passes through the gut (Lakshmi et al. 2013). Currently, major efforts are being made to replace inactivated or attenuated vaccines by safer and more effective subunit vaccines (Liljeqvist and Ståhl 1999). In addition, several approaches are being used in the development of a new generation of vaccines able to confer protection against oral-entry parasites. This type of vaccine requires the induction of protection at the mucosal level and cell-mediated responses. In this context, antigen production from different plant platforms has aroused a great interest as an alternative strategy to develop safer and affordable subunit vaccines (Paul and Ma 2010). In fact, a large number of antigens derived from viruses or bacteria have been transiently expressed in transgenic plants and in plant cell cultures (Yusibov et al. 2011).

The main vaccine antigens expressed in plants include both the antigens for mucosal vaccines against diseases, such as diarrhea, hepatitis B, and rabies, and the antigens for injectable vaccines against diseases, such as non-Hodgkin's lymphoma,

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influenza A virus H1N1 and H5N1, and Newcastle disease (Paul and Ma 2010). Interestingly, many of these antigens have proved to be efficient and safe in animal models and preclinical trials (Yusibov et al. 2011). In fact, Dow AgroSciences (http://www.dowagro.com/) has developed the first vaccine for Newcastle disease produced in plants and approved to be used on chickens. These data stimulate to continue improving plant expression systems to turn them into competitive systems for the generation of new vaccines against different pathogens.

Epidemiological Impact of Toxoplasmosis

Toxoplasmosis is a disease caused by the protozoan parasite *Toxoplasma gondii* (Montoya and Liesenfeld 2004). This obligate intracellular parasite belongs to the phylum Apicomplexa, class Sporozoea, subclass Coccidia (Levine 1977), and is one of the most successful parasites widely distributed and able to infect all warm-blooded animals, including humans (Dubey 2008).

The three infective stages of *T. gondii* are known as: (1) sporozoites: proliferative forms that develop on the inside of the oocysts released in the feces of cats; (2) tachyzoites: stage where the parasite has high replicative capacity (acute or active infection); and (3) bradyzoites: forms that develop within cysts in the tissues of infected animals, where the parasite has low replicative capacity (chronic and latent infection). The parasite's life cycle includes two phases: (1) a sexual phase, which occurs only in the intestinal epithelium of cats during the acute phase of infection and results in the production of oocysts/sporozoites and (2) an asexual phase, which occurs in all tissues of infected birds and mammals, including cats, and involves tachyzoites and bradyzoites (Fig. 11.1). Normally, *T. gondii* is transmitted by ingestion of cysts found in the tissues of infected animals or by ingestion of food or water contaminated with oocysts released in the feces of infected cats (Dubey 2000, 2008).

Oocysts contain the parasite in its sporozoite stage, while tissue cysts contain the latent form of bradyzoites. These two forms of the parasite enter the body by oral ingestion, invade the intestinal epithelial cells, and change to the highly replicative form (tachyzoites), invading all tissues, mainly those of the nervous system. Tachyzoite proliferation, known as the acute infection, occurs not only in the intermediate hosts (mammals and birds) but also in the definitive host (cats). Tachyzoites can enter any type of cells of the host and divide actively until the cell dies, releasing more tachyzoites (Robert-Gangneux and Dardé 2012). This stage causes tissue necrosis. Tachyzoites remain longer in the spinal cord and brain, because the immune response is less effective in this kind of tissue (Burg et al. 1988). The chronic state of the infection begins 15 days after the entry of the parasite into the organism. During this state, the tachyzoites present in the tissue cysts change to bradyzoites and disappear from the visceral tissue (Weiss and Kim 2000). The parasite then enters a "repose stage," in which the tissue cysts are usually located in the brain, liver, and muscle (Robert-Gangneux and Dardé 2012). These cysts cause no apparent effect on the host and can stay there for the rest of the life of animals and humans (Hill and Dubey 2002).

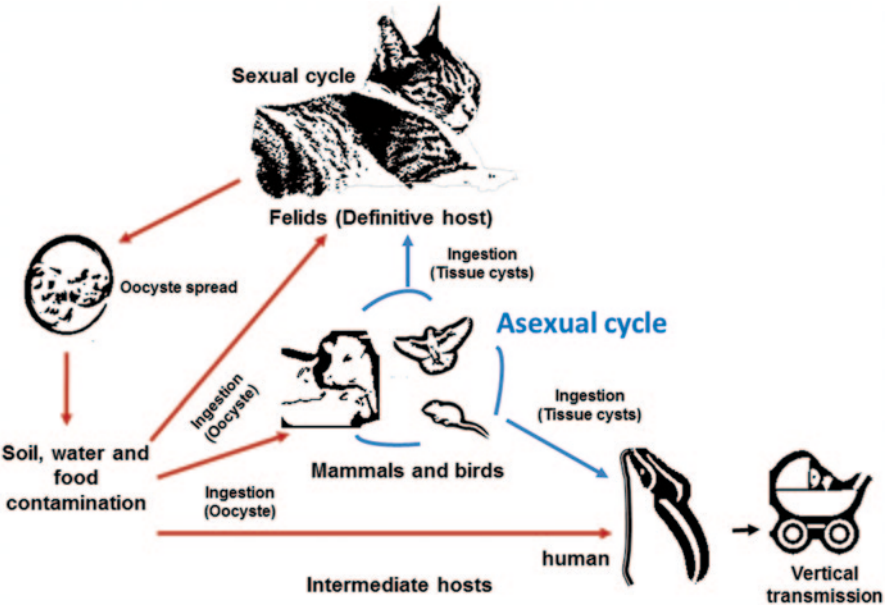

Fig. 11.1 The life cycle of *Toxoplasma gondii*

Toxoplasmosis is present at all latitudes, not only in human populations but also in more than 300 species of domestic and wild mammals and in about 30 species of poultry and wild birds (Hill and Dubey 2002; Innes 2010). The incidence of *Toxoplasma* infection may change according to the environmental conditions, cultural habits, and presence of animal species, and even in different regions of a same country (Petersen et al. 2010). In humans, toxoplasmosis is widely distributed around the world. It is generally assumed that approximately 25–30% of the world's human population is infected by *Toxoplasma* (Montoya and Liesenfeld 2004). Humans become infected by ingesting soil or water contaminated with oocysts, from tissue cysts in undercooked meat, and other less common means (Robert-Gangneux and Dardé 2012). Direct contact with cats does not appear to pose a significant risk of infection in humans, given that oocysts are not infective when passed from cats to humans, and the duration of oocyst shedding is short (Kijlstra and Jongert 2008; Petersen et al. 2010). In the USA and the UK, the rate of infection in human populations varies between 16 and 40%. On the other hand, in Latin America (due to the consumption of contaminated water) and Europe (due to the consumption of uncooked meat), the prevalence of toxoplasmosis varies between 50 and 80% (Montoya and Liesenfeld 2004; Pappas et al. 2009). In fact, in Europe, recent data have shown that toxoplasmosis is one of the most important causative agents of food-borne diseases (Kortbeek et al. 2009).

In humans, most of the patients infected with *T. gondii* exhibit no clinical symptoms. However, when these patients have deficiencies in the immune system, they may experience reactivations of bradyzoites to tachyzoites, with serious

consequences (Henriquez et al. 2010). In humans, toxoplasmosis is known for its severe sequelae, such as those causing lethal encephalitis in immunocompromised patients as those with acquired immunodeficiency syndrome (AIDS; Luft and Remington 1992; Velge-Roussel et al. 1994). About 45 % of AIDS patients develop encephalitis by toxoplasmosis, which results in 10–30 % mortality as a result of uncontrolled *T. gondii* infection (Luft and Remington 1992; Henriquez et al. 2010). In addition, when pregnant women are infected with *T. gondii* for the first time, they can transmit toxoplasmosis to their fetuses, inducing abortions or causing hydroencephalitis or uveitis in their newborns (Mitchell et al. 1990; Minkoff et al. 1997). When primary infection is acquired by a pregnant woman, tachyzoites can colonize placental tissues during the dissemination process and can then gain access to the fetal compartment in about 30 % of cases (Robert-Gangneux and Dardé 2012). Villena et al. (2010) reported that the prevalence of congenital toxoplasmosis in France is around 3.3 per 10,000 live births. This value is similar to that reported in Brazil (1 per 3,000 live births), whereas in the USA a pilot study in Massachusetts showed a prevalence of 1 per 10,000 live births (Guerina et al. 1994). In the UK, Stanford et al. (2006) showed that 50 % of children with chorioretinitis develop this condition due to *Toxoplasma* infection after birth. In addition, Holland (2003) estimated that approximately 2 % of infected individuals will have ocular involvement.

Up to now, it was thought that chronic toxoplasmosis represented no risk for the infected individual. However, recently, chronic infections by *T. gondii* have been associated with different diseases or disorders associated with the nervous system, such as brain tumors, attention deficit, hyperactivity, compulsive obsession, and schizophrenia (Brynska et al. 2001; Yolken et al. 2009; Miman et al. 2010; Pedersen et al. 2011; Thomas et al. 2012; Vittecoq et al. 2012).

In addition, *Toxoplasma* infection has high incidence in important livestock animals. Mainly, the congenital infection with *T. gondii* has high prevalence in sheep and goats (Tenter et al. 2000). In fact, toxoplasmosis is one of the main causes of reproductive failures in these animals, thus leading to great economic losses in livestock (Innes and Vermeulen 2006). For example, in the UK, Menzies et al. (2008) estimated that 1.2–2.2 % of a total of 16 million sheep lose their fetuses due to *Toxoplasma* infection. On the other hand, in Europe and the USA, *T. gondii* is transmitted to humans mainly by ingestion of uncooked meats from infected pigs and sheep (Tenter et al. 2000; Jones and Dubey 2012). In fact, Cook et al. (2000) estimated that consumption of not-well-cooked meat was the cause of infection in 30–60 % of pregnant women with acute toxoplasmosis. These great global important aspects for public health and economy make the development of an effective vaccine against toxoplasmosis a goal of great relevance.

The S48 strain of live attenuated tachyzoites is currently the only commercial vaccine for use in sheep (Buxton et al. 1991). This live vaccine (Toxovax) is commercially marketed in the UK, France, and New Zealand to reduce losses to the sheep industry from congenital toxoplasmosis (Buxton and Innes 1995). The vaccine consists of a modified strain (S48) of *T. gondii,* originally isolated from an aborted lamb in New Zealand. By repeated passage in mice for many years, the strain lost the capacity to form tissue cysts and oocysts. The commercial vaccine

consists of live cell culture-grown tachyzoites that have a shelf life of 10 days. It is recommended to be given 3 weeks before mating. Vaccination of sheep with S48 allows decreasing abortions by 75 %, decreasing neonatal mortality, and improving the weight at birth (Buxton et al. 1991). However, this vaccine causes side effects, induces brief immunity, and has a short shelf life and a high cost. Moreover, this type of live vaccine has the risk of reverting to a pathogenic strain, thus not being suitable for human use (Kur et al. 2009). On the other hand, in humans, there are some drugs that are effective during the acute phase of the infection, but whose implementation is complicated because the primary infection is difficult to diagnose. The treatment with these available drugs is also difficult due to their toxic effects and rapid reinfection (Buxton and Innes 1995). For all these reasons, and considering that vaccination against *T. gondii* is the most efficient and safe method to prevent this infectious disease, current researches are focused on the development of purified or recombinant parasite proteins or DNA vaccines by evaluating antigen combinations, adjuvants, and inoculation routes that prevent congenital and/or acquired *Toxoplasma* infection.

Vaccination Targets in Toxoplasma Subunit Vaccines

Developing a vaccine against *T. gondii* is important because of the implications of this disease not only on human health but also on the economy (Cenci-Goga et al. 2011). Taking into account the main sources and transmission routes of *T. gondii,* a vaccine against toxoplasmosis in humans should prevent infection of pregnant women to avoid congenital toxoplasmosis, reducing costs due to the rigorous following up of at-risk pregnant women, and control parasite reactivation in immunocompromised patients to avoid the use of toxic drugs during toxoplasmosis treatment (Mui et al. 2008), whereas a vaccine against toxoplasmosis in animals should induce livestock protection to reduce economic losses and control a transmission vector to humans through safe meats, and promote immunity in domestic cats to avoid the environmental contamination with oocysts and the infection risk of intermediate hosts (Innes et al. 2009).

T. gondii infection induces specific antibody response and a strong cell type T helper (Th1) response characterized by IFN-γ production (Henríquez et al. 2010). IFN-γ is required to control tachyzoite proliferation during the acute stage of *T. gondii* infection. In fact, the protective activity of T cells is mediated predominantly by IFN-γ. In addition, both CD4+ and CD8+ cells play an important role during the protection against *T. gondii,* which is correlated with IFN-γ production and CD8+ cytotoxic T cell response restricted to the major histocompatibility complex class I (MHC-I) in both humans and mice (Subauste et al. 1991; Montoya et al. 1996). CD8+ cytotoxic T cells are the main cells responsible for a protective immune response, since these cells are involved in the control of infected cells during the acute phase and of the number of cysts during the chronic phase of the infection (Henriquez et al. 2010). For this reason, the ideal vaccine against toxoplasmosis

should include specific antigens capable of inducing a Th1 immune response with IFN-γ production and CD8+ stimulation.

The tachyzoites of the RH strain are frequently used to study the antigen properties of *T. gondii* (Nguyen et al. 2003). In recent years, significant progress has been made in the identification of vaccine candidates capable of inducing a protective immune response. Most of the researches on the antigenic structure of *T. gondii* have been focused on membrane surface antigens and antigens released by secretory organelles (Capron and Dessaint 1988; Cesbron-Delauw and Capran 1993; Cesbron-Delauw 1994; Bourguin et al. 1993; Saavedra et al. 1996; Huynh et al. 2003; Lebrun et al. 2005; Mercier et al. 2002).

SAG1, SAG2, and SAG3 are the major surface antigens of tachyzoites (Couvreur et al. 1988); among them, SAG1 is one of the predominant vaccine candidates (Petersen et al. 1998; Chen et al. 2002; Couper et al. 2003; Zhou et al. 2007; Qu et al. 2008; Li et al. 2011; Dziadek et al. 2011). Excretory secretory antigens are expressed by both tachyzoites and bradyzoites (Capron and Dessaint 1988; Cesbron-Delauw and Capran 1993). The main components of excretory secretory antigens are dense granule antigen (GRA) molecules (Cesbron-Delauw 1994). GRA4 and GRA7 have been identified as leading vaccine candidates (Desolme et al. 2000; Vercammen et al. 2000; Martin et al. 2004; Mévelec et al. 2005; Jongert et al. 2007; Jongert et al. 2008; Chen et al. 2009; Hiszczynska-Sawicka et al. 2010, 2011; Min et al. 2012).

SAG1

SAG1 is a stage-specific antigen that participates in the cell invasion process. SAG1 is only detected in the membrane of tachyzoites and comprises between 3 and 5% of the total proteins of the parasite (Kasper et al. 1983, 1984). The SAG1 amino acid sequence presents 12 cysteine residues involved in the formation of disulfide bridges and correct protein folding (Burg et al. 1988). Inside the parasite, previous to anchorage to the tachyzoite membrane, the proSAG1 protein (of 336 amino acids) suffers a series of posttranslational modifications: It loses the first 78 amino acids (signal peptide) from the N-terminal end and has a hydrophobic C-terminal region which is cleaved, whereas a glycosylphosphatidylinositol (GPI) domain is added to the resulting SAG1 polypeptide (Chen et al. 2001).

SAG1 is an excellent candidate for the immunoprophylaxis for toxoplasmosis since it presents a low polymorphism between different *T. gondii* strains (Lekutis et al. 2001), and induces a strong specific antibody response (Kasper et al. 1983). On the other hand, SAG1 seems to be capable of stimulating IFN-γ production by T cells in seropositive individuals (Khan et al. 1988). In addition, numerous studies have shown SAG1 potential as a vaccine as purified protein, recombinant protein, or DNA vaccine (Table 11.1). Initially, Petersen et al. (1998) showed that immunization with recombinant SAG1 in alum induced partial protective immunity against lethal infection with *T. gondii* in mice. Later, Liu et al. (2006) showed that

Table 11.1 Examples of immunization experiments with *Toxoplasma gondii* SAG1 antigen

Antigen	Animal	Immunization/adjuvant	Challenge	Results	References
SAG1-*E. coli*	NMRI mice	Subcutaneously/Alum	*T. gondii* RH strain	Prolonged survival time. High levels of IgG1	Petersen et al. (1998)
SAG1-plasmid encoding gene	C3H and BALB/c mice	Intramuscularly	10^5 tachyzoites of *T. gondii* RH strain	Reduction of brain cysts (80–100%). High levels of IFN-γ. CD8+ contributed to protective immunity	Nielsen et al. (1999)
SAG1-plasmid encoding cDNA	C57BL/6 mice	Intramuscularly/GM-CSF	80 cysts of *T. gondii* ME49 strain	Protection against acute toxoplasmosis. High levels of IFN-γ and IL-2	Angus et al. (2000)
SAG1-*P. pastori*	Dunkin–Hartley guinea pigs	Subcutaneously/SBAS1	10^4 tachyzoites	Reduction of parasites in fetuses (66–86%)	Haumont et al. (2000)
SAG1 purified from *T. gondii*	CBA/J mice	Intranasally/cholera toxin	Cysts of *T. gondii* 76K strain	Reduction of brain cysts (50-60%). Strong specific proliferative response in mucosal compartments	Velge-Roussel et al. (2000)
SAG1 purified from *T. gondii*	CBA/J mice	Intranasally/cholera toxin and heat-labile enterotoxin	70 cysts of *T. gondii* 76K strain	High levels of systemic IgG and mucosal IgA. High levels of IFN-γ and IL-2 in splenocytes and IL-2 in mesenteric lymph node cells	Bonenfant et al. (2001)
SAG1-plasmid encoding gene	BALB/c mice	Intramuscularly	10–20 cysts of a *T. gondii* virulent Beverley strain	Increased survival rates (66.7%). High levels of IgG2a and IFN-γ	Couper et al. (2003)
SAG1/SAG2-*S. typhimurium*	BALB/c mice	Intragastric/A_2/B subunits of cholera toxin	10^3 tachyzoite of *T. gondii* RH strain	Increased survival rates (40%). Induction of humoral and Th1 type cellular immune responses	Cong et al. (2005)

Table 11.1 (continued)

Antigen	Animal	Immunization/adjuvant	Challenge	Results	References
SAG1-*E. coli*	BALB/c mice	Intradermally/Freund	10^5 tachyzoite of *T. gondii* RH strain	Delayed death for 60 h. High titers of IgG and IgM, IFN-γ, IL-2, and IL-4	Liu et al. (2006)
SAG1/MIC4-plasmid encoding gene	BALB/c mice	Intranasally/A_2/B subunits of cholera toxin	10^3 tachyzoite of *T. gondii* RH strain	Increased survival rates (14%) and prolong the life of mice. High levels of IgG2a, IFN-γ and IL-12	Wang et al. (2009)
SAG1/ROP2-plasmid encoding gene	BALB/c mice	Intramuscularly/Freund	10^4 tachyzoite of *T. gondii* RH strain	Increased survival rates. High levels of IFN-γ, TNF-α and IL-2	Hoseinian Khosroshahi et al. (2011)
SAG1/MIC3-*Baculovirus*	BALB/c mice	Intramuscularly	10^3 tachyzoite of *T. gondii* RH strain	Increased survival rates (50%). High levels of anti-*T. gondii* TLA antibodies, proliferation and IFN-γ	Fang et al. (2012)
SAG1/14–3-3- plasmid encoding gene	BALB/c mice	Intramuscularly	10^4 tachyzoite of *T. gondii* RH strain	Increased survival time. Higher levels of IgG2a than IgG1. High levels of IFN-γ	Meng et al. (2012)
SAG1/SAG3- plasmid encoding gene	BALB/c mice	Intramuscularly/A_2/B subunits of cholera toxin	10^3 tachyzoite of *T. gondii* RH strain	Increased survival rates (20–40%). High levels of anti-*T. gondii* IgG, IFN-γ, lymphocyte proliferation and CD8+ T cells	Cong et al. (2013)
SAG1-viral vector (adenovirus and Ankara virus)	C57BL/6 mice	Subcutaneously and intramuscularly	10 cysts of *T. gondii* ME49 strain	Increased survival rates (60%). Reduction of brain cysts (50%). High levels of IgG1 and IgG2c, IFN-γ and TNF-α. Production of CD8+ cells	Mendes et al. (2013)

recombinant SAG1 induces a dominant antibody response and a strong Th1 T cell response characterized by high titer of IFN-γ production, suggesting that SAG1 is a good vaccine candidate to control toxoplasmosis. In addition, Haumont et al. (2000) evaluated the protective immunity against congenital toxoplasmosis with recombinant SAG1 in a guinea pig model. These authors demonstrated a reduced parasitic load (from 66 to 86%) in fetuses derived from adult guinea pigs immunized with SAG1 and challenged with the virulent *T. gondii* C56 strain, suggesting that SAG1 elicits a significant protection against vertical transmission. In addition, Velge-Roussel et al. (2000) and Bonenfant et al. (2001) performed intranasal (i.n.) immunization using SAG1 plus heat-labile toxin or cholera toxin as adjuvants in a murine model and showed that mucosal *T. gondii* invasion of the host can be partially controlled. On the other hand, also using a murine model, other researchers have shown that DNA vaccines based on SAG1 exert a high degree of protection against lethal challenges, correlating such protection with the induction of a cytotoxic response T lymphocyte (Nielsen et al. 1999; Angus et al. 2000; Couper et al. 2003; Liu et al. 2010).

Vector vaccines based on *Salmonella typhimurium* (Cong et al. 2005), adenovirus (Caetano et al. 2006), and pseudorabies (Liu et al. 2008) expressing the SAG1 have also been used. All of them have shown a high protective response against *Toxoplasma* infection. Also, different prime-boost strategies using the SAG1 antigen, in combination with recombinant protein and DNA vaccine, have shown to be highly effective in enhancing immune responses against *T. gondii* infection (Shang et al. 2009; Li et al. 2011). Finally, the SAG1 has also been used as a multi-antigen vaccine as recombinant protein or DNA vaccine in mice (Fachado et al. 2003; Hoseinian Khosroshahi et al. 2011; Fang et al. 2012; Cong et al. 2013). In all cases, the results have shown that a multi-antigen vaccine strategy induces a greater cell and antibody response than a single-antigen vaccine (Hoseinian Khosroshahi et al. 2011; Fang et al. 2012; Cong et al. 2013). Taken together, these antecedents show that SAG1 is able to develop significant protection against *Toxoplasma* infection in animal models, supporting the idea that this antigen can be used in the development of anti-*T. gondii* vaccines.

GRA4

GRA4 participates in the parasite–host interaction, forming a stable complex with other proteins like GRA6 and GRA2, involved in the transport of nutrients and proteins into vacuoles (Labruyère et al. 1999). The coding sequence of GRA4 (345 amino acids) contains a putative N-terminal signal (around 20 amino acids). Also, GRA4 presents a high proline content (12%), an internal hydrophobic region (19 amino acids) near the C-terminal region, and a potential N-glycosylation site (Mévelec et al. 1992, 1998). The region between amino acids 229 and 249 has been identified as a region that induces T cell proliferation in splenocytes of CBA/J *T. gondii*-infected mice, suggesting that this region is involved in the T cell re-

sponse (Mévelec et al. 1998). On the other hand, another region called the C domain (between amino acids 297 and 345) is strongly recognized by immunoglobulin G (IgG) and immunoglobulin A (IgA) antibodies from sera and intestine, respectively, and antibodies from milk of *T. gondii*-infected mice (Mévelec et al. 1992, 1998). In fact, Mévelec et al. (1998) identified two B epitopes in the C domain: one situated in the last 11 amino acid residues in the C-terminal region, and the other located between amino acids 318 and 334 of the GRA4 protein (Mévelec et al. 1998).

Based on these characteristics, different researchers have assayed GRA4 in immunization protocols in the murine model (Table 11.2). They showed that GRA4 was able to elicit both mucosal and systemic immune response after *T. gondii* oral infection (Desolme et al. 2000; Martin et al. 2004; Mévelec et al. 2005; Chen et al. 2009; Sánchez et al. 2011). They concluded that mouse immunization with the recombinant GRA4 protein or with the coding gene confers high protection against *Toxoplasma* infection (Desolme et al. 2000; Martin et al. 2004, Mévelec et al. 2005; Chen et al. 2009). These authors also observed that the recombinant GRA4 protein or GRA4 DNA vaccines are able to induce a Th1-specific humoral and cellular response with production of IFN-γ (Martin et al. 2004; Mevelec et al. 2005; Chen et al. 2009). In addition, Mevelec et al. (2005) evaluated the effect of GRA4 as DNA vaccine to prevent congenital infection and found a high survival of newborns from GRA4-immunized outbred mice exposed to infection during gestation. Finally, other authors have used GRA4 in the development of a multi-antigen vaccine (Sanchez et al. 2011; Dziadek et al. 2012). In particular, Dziadek et al. (2012) showed that recombinant antigen cocktails that include GRA4 are very effective in the development of a high level of protection, independently of the genetic backgrounds and innate resistance to toxoplasmosis of the mouse strain, and suggested that GRA4 may be considered as a feasible candidate in vaccine development.

GRA7

GRA7 from dense granules has molecular mass of approximately 29 kDa (Jacobs et al. 1998). This protein is important in cell invasion, maintenance of the parasitophorous vacuole, and survival of the parasite after cellular invasion (Carruthers et al. 1999). The coding sequence of GRA7 contains a putative N-terminal signal peptide, one site of potential N-linked glycosylation, and a C-terminal region containing two hydrophobic regions, one of which has the characteristics of a putative transmembrane domain (Fischer et al. 1998; Jacobs et al. 1998). Among GRA proteins, GRA7 is the only one that is expressed in all infectious forms of *T. gondii* (tachyzoites, bradyzoites, and sporozoites), suggesting a particular importance in the parasite's intracellular life phase (Ferguson et al. 1999; Neudeck et al. 2002). On the other hand, a T cell response against GRA7 has been observed in chronically infected individuals. In addition, human B cell epitopes have also been identified in this protein (Jacobs et al. 1999). The three GRA7 epitopes defined by murine antibodies are located on the N-terminal segment of the putative transmembrane

Table 11.2 Examples of immunization experiments with *Toxoplasma gondii* GRA4 antigen

Antigen	Animal	Immunization/adjuvant	Challenge	Results	References
GRA4-plasmid encoding gene	C57BL/6 mice	Intramuscularly/IL-12	40 cysts of *T. gondii* 76K strain	Increased survival rates (62%). High levels of anti-GRA4 IgG2b, IFN-γ and IL-10	Desolme et al. (2000)
GRA4/ROP2-*E. coli*	C57BL/6 and C3H mice	Intramuscularly/alum	20 or 100 cysts of *T. gondii* ME49 strain	No differences in the survival rates. Reduction of brain cysts. Higher levels of IgG1 than IgG2a. High levels of IFN-γ	Martin et al. (2004)
GRA4/SAG1-plasmid encoding gene	C57BL/6 mice	Intramuscularly/GM-CSF	40 cysts of *T. gondii* 76K strain	Increased survival rates in adults (62–87%) and pups (50%). Reduction of brain cysts (90%) in pregnant female and pups	Mévélec et al. (2005)
GRA4-plasmid encoding gene/GRA4-vaccinia virus	C57BL/6 mice	Intramuscularly	20,000 *T. gondii* tachyzoites	Increased survival rates (90%). Higher levels of anti-*T. gondii* IgG1 than IgG2a. High levels of IFN-γ	Zhang et al. (2007)
GRA4-plasmid encoding gene	C57BL/6 and BALB/c mice	Intramuscularly/ liposome-encapsulation	20 or 80 cysts of ME49 *T. gondii* strain or 10^3 tachyzoites of RH *T. gondii* strain	Increased survival rates (54.5%) in C57BL/6 mice. Prolonged life in BALB/c mice. High levels of IFN-γ and IL-2	Chen et al. (2009)
GRA4/ROP2-*E. coli*	C3H mice	Intramuscularly/CpG	20 cysts of *T. gondii* ME49 strain	Reduction of brain cysts (63%). Higher levels of IgG2a than IgG1. High levels of IFN-γ	Sanchez et al. (2011)
GRA4/SAG1/ROP2/ ROP4-*E. coli*	BALB/c mice	Subcutaneously/Freund	5 cysts of *T. gondii* DX strain	Reduction of brain cysts (77–84%). Higher levels of anti- *T. gondii* IgG1 than IgG2a. High levels of IFN-γ and IL-2	Dziadek et al. (2012)

domain, this region being a major immunogenic domain (Neudeck et al. 2002). On the other hand, GRA7 is recognized by serum antibodies from *T. gondii*-infected humans. In fact, a strong antibody response against GRA7 has been found in human sera from acute infected patients (Pfrepper et al. 2005). Also, GRA7 is a target of the intracerebral immune response during the chronic phase of infection (Vercammen et al. 2000; Neudeck et al. 2002). Based on this, different researchers have evaluated GRA7 as a vaccine candidate for the prevention and control of toxoplasmosis (Table 11.3).

Initially, Vercammen et al. (2000) demonstrated that vaccination with plasmids encoding *T. gondii* antigens GRA7, GRA1, or ROP2 (rhoptry 2) exerted a similar degree of protection in different mouse strains. Later, Jongert et al. (2007) evaluated the contribution of each antigen in the humoral and cellular immune response elicited after vaccination with DNA vaccine cocktails containing GRA1, GRA7, and ROP2 genes and found that reductions in brain cysts were observed only after vaccination with the mixture containing GRA1 and GRA7 or GRA7 and ROP2, but not with that containing GRA1 and ROP2. In particular, a reduction of 89 % in a brain cyst load in mice was achieved with the administration of a GRA1–GRA7 cocktail DNA vaccine. In addition, in low-dose single-gene vaccinations, IFN-γ production and strong protection were induced only by GRA7, suggesting that GRA7 is the main component in the multigene vaccine for vaccination against toxoplasmosis. In addition, Jongert et al. (2008) demonstrated that intradermal immunization with a GRA1–GRA7 cocktail DNA vaccine is able to elicit a strong humoral and Th1 cellular immune response characterized by IFN-γ production against *Toxoplasma* infection in pigs. More recently, Hiszczynska-Sawicka et al. (2011) evaluated the immune responses of sheep injected intramuscularly with DNA plasmids encoding *T. gondii* dense granule antigens GRA1, GRA4, GRA6, or GRA7 formulated into liposomes and demonstrated that the plasmid DNA encoding GRA7 was the most effective vaccine to stimulate an immune response in sheep. In addition, the immune response was correlated with strong IFN-γ and IgG2 responses against the injected GRA7 antigen (Hiszczynska-Sawicka et al. 2011). These results suggest that GRA7 may be a good choice for the development of vaccine formulations against toxoplasmosis in ovine.

Plant-Based Vaccines Developed Against Toxoplasmosis

Since the portal of entry of *T. gondii* is the mucosa, an efficient stimulation of the mucosa and an adequate systemic response constitute a priority which could be accomplished by the administration of an oral or nasal vaccine (Bout et al. 2002, García et al. 2007). Therefore, *Toxoplasma* infection is an interesting model to study the optimization of expression systems based on plants for the production of eukaryotic immunoprophylactic antigens. In addition, *T. gondii* is an excellent model to assess the effectiveness of oral vaccines against intracellular pathogens because *Toxoplasma* infection has been well characterized in the murine model.

Table 11.3 Examples of immunization experiments with *Toxoplasma gondii* GRA7 antigen

Antigen	Animal	Immunization/adjuvant	Challenge	Results	References
GRA7/GRA1-plasmid encoding gene	C57BL/6, BALB/c, and C3H mice	Intramuscularly	10 or 25 cysts of *T. gondii* 76K strain	Increased survival rates (50–90%). Higher titers of IgG2a than IgG1. High levels of IFN-γ	Vercammen et al. (2000)
GRA7/GRA1/ROP2-plasmid encoding mature protein	C3H mice	Intramuscularly	20 cysts of *T. gondii* 76K strain	Reduction of brain cysts (89%). Higher titers of IgG2a than IgG1. High levels of IFN-γ	Jongert et al. (2007)
GRA7/GRA5/ROP2-*E. coli*	BALB/c mice	Intranasally/cholera toxin	*T. gondii* RH strain	Reduction of brain cysts (58%). High titers of IgG against rROP2 and rGRA7	Igarashi et al. (2008)
GRA7/GRA1-plasmid encoding mature protein	Bred pigs	Intradermally/GM-CSF	4×10^3 live *T. gondii* RH tachyzoites + CpG	High levels of IgG against rGRA7 and rGRA1. High levels of IFN-γ after challenge	Jongert et al. (2008)
GRA7-plasmid encoding gene	Ewes	Intramuscularly/liposomes + CpG	_____	High levels of IgG2 and IFN-γ	Hiszczynska-Sawicka et al. (2010)
GRA7/GRA1/GRA4/GRA6-plasmid encoding gene	Ewes	Intramuscularly/liposomes + CpG	_____	High levels of IgG2 anti-GRA7 and IFN-γ	Hiszczynska-Sawicka et al. (2011)
GRA7-plasmid encoding gene and GRA7-*E. coli*	BALB/c mice	Intramuscularly and subcutanously/Freund	1,000 *T. gondii* tachyzoites	Increased survival rates (60%). High levels of IgG2a and IFN-γ	Min et al. (2012)
GRA7/ROP1-plasmid encoding gene	BALB/c mice	Intramuscularly/IL-12	Cysts of *T. gondii* ME49 strain	Increased survival rates (33–50%). Higher titers of IgG2a than IgG1. High levels of IFN-γ, TNF-α and IL-10	Quan et al. (2012)

Our research group was the first to express the mature SAG1 protein of *T. gondii* (residues 77 to 336, excluding the signal peptide), which was the first *Toxoplasma* antigen expressed in plants (Clemente et al. 2005). The *Agrobacterium*-mediated transient expression system was used to test the expression level of three constructs carrying $SAG1_{77–336}$. Two constructs were based on a potato virus X (PVX) amplicon, whereas the other was based on the *SAG1* gene fused to an apoplastic peptide signal under the *Cauliflower mosaic virus* (CaMV) 35S promoter. $SAG1_{77–336}$ (35 kDa) accumulation in leaves ranged from 0.1 to 0.06% of total soluble protein (equivalent to 1 and 0.6 μg of $SAG1_{77–336}$ per gram of fresh weight, respectively). The SAG1 accumulation levels were slightly higher with amplicons. The better replication capacity of amplicons could explain the higher SAG1 levels compared with the construct where the *SAG1* gene was fused to an apoplastic peptide signal. Afterwards, in order to improve the nuclear expression of SAG1 in plants, we evaluated codon-optimized SAG1 genes and plant cell compartment-targeting signal sequences by using vacuum agroinfiltration in tobacco leaves (Laguía-Becher et al. 2010). In addition, we deleted 14 hydrophobic C-terminal residues that are processed in the native SAG1 protein. The resulting unmodified $SAG1_{77–322}$ gene accumulated five to ten fold more than leaves agroinfiltrated with a codon-optimized $SAG1_{77–322}$ gene, suggesting that modifications in the $SAG1_{77–322}$ sequence could have affected the rate of translation. On the other hand, for protein-stable accumulation, we fused the plant-optimized $SAG1_{77–322}$ gene and the native $SAG1_{77–322}$ gene to the apoplastic peptide signal or to the endoplasmic reticulum peptide signal (Laguía-Becher et al. 2010). The results showed that the levels of the endoplasmic reticulum-localized native protein were 50% higher than those obtained by apoplast-localized native protein. This indicates that the retention of $SAG1_{77–322}$ protein in the endoplasmic reticulum was highly positive, and low expression levels of the optimized $SAG1_{77–322}$ version in the reticulum would be restricted to the efficiency of the translation. In addition, $SAG1_{77–322}$ yield was lower in the cytoplasm using PVX amplicon than in the endoplasmic reticulum using nuclear expression systems (Laguía-Becher et al. 2010). On the other hand, the removal of the C-terminus did not affect $SAG1_{77–322}$ accumulation, when compared with its full-length protein (Laguía-Becher et al. 2010). Together, these results show that only the accumulation of the native $SAG1_{77–322}$ protein in the endoplasmic reticulum enhances the expression levels of this antigen expressed in plants (1.3 μg per gram of fresh weight; Laguía-Becher et al. 2010).

GRA4 is another antigen that has also been expressed in plant (Ferraro et al. 2008; De L Yácono et al. 2012). Initially, the truncated GRA4 ($GRA4_{183–345}$) sequence (Martin et al. 2004) was chosen for transient expression in plants, based on a PVX amplicon, which allows cytoplasmic accumulation of the recombinant protein. An alternative strategy for plant expression was investigated for antigen secretion into the extracellular space (Ferraro et al. 2008). The yields of $GRA4_{183–345}$ in infiltrated tobacco leaves were in the order of 0.01% of total soluble protein, which represents around 0.2 μg/g of fresh weight (Ferraro et al. 2008). In addition, the $GRA4_{183–345}$ protein was also detected in the apoplastic washing fluids, suggesting that the recombinant protein was efficiently targeted to the apoplasmic space (Ferraro et al. 2008).

In order to improve the GRA4 expression level in plants, De L Yácono et al. (2012) also expressed $GRA4_{183–345}$ in transplastomic plants. Chloroplast transformation offers many advantages, including high levels of transgene expression likely due to a high copy number, absence of epigenetic effects, transgene containment via maternal inheritance, and multigene expression in a single transformation event (Lössl and Waheed 2011). De L Yácono et al. (2012) found that chloroplast $GRA4_{183–345}$ expression levels in the transplastomic plants were up to 6 μg/g of fresh weight (or 0.2% of total protein). Although chloroplast $GRA4_{183–345}$ levels were not as high as the usual ones in plastid transformation, chloroplast transformation allowed a significant 30-fold increase in GRA4 protein accumulation in the plant (De L Yácono et al. 2012).

Studies on the immunogenicity of these plant-made *T. gondii* antigens have shown that SAG1 and GRA4 expressed in plants are able to elicit an immune response by subcutaneous or oral vaccination in a murine model (Clemente et al. 2005; Laguía-Becher et al. 2010; De L Yácono et al. 2012). These findings provide a rationale for the development of a plant-made oral vaccine against toxoplasmosis. We demonstrate the immunogenic properties of plant-derived SAG1 (Clemente et al. 2005), finding that subcutaneous immunization with SAG1 transiently expressed in plants in the presence of incomplete Freund's adjuvant induced a significant increase in the systemic-specific antibodies and a partial protection against anon-lethal challenge with *T. gondii* (Clemente et al. 2005). Later, we characterized the immune response elicited by the extract from leaves infiltrated with the native SAG1 fused to the endoplasmic reticulum peptide signal and found that leaf-SAG1 immunization elicited a Th1 cellular response characterized by significant IFN-γ production in splenocyte supernatants (Laguía-Becher et al. 2010). In addition, in order to improve the immunoprotection of plant-derived SAG1, we included a prime-boost protocol with a recombinant SAG1 expressed in bacteria (rSAG1) (Laguía-Becher et al. 2010). The protection was increased when mice were intradermally boosted with rSAG1 (SAG1 + boost) (Fig. 11.2). In addition, these mice elicited a significant Th1 humoral and cellular immune response characterized by high levels of IFN-γ (Fig. 11.3). We also tested whether leaf-SAG1 oral vaccination could positively influence the outcome of *T. gondii* infection without the use of any adjuvant. In the oral immunization assay, the SAG1 + boost group showed a significantly lower brain cyst burden than the rest of the groups, which correlated with an increased humoral response (Fig. 11.4), suggesting that immunization with tobacco leaves expressing SAG1 is a reliable system to generate immunity and that it could be boosted by heterologous prime-boost inoculation regimens (Laguía-Becher et al. 2010).

Since GRA4 is a target antigen for both mucosal and systemic immune responses, De L Yácono et al. (2012) evaluated the immunogenicity of chloroplast-derived $GRA4_{183–345}$ protein in an oral vaccination approach without the use of any adjuvant. It was found that oral administration of adjuvant-free chlGRA4 was enough to elicit an immune response capable of inducing partial protection against *T. gondii* infection measured as a marked reduction in brain cyst loads (Fig. 11.5). In addition, we showed that ChlGRA4 immunization elicited both a mucosal immune response, characterized by the production of specific IgA, and IFN-γ, interleukin-4 (IL-4), and IL-10 secretion by mesenteric lymph node cells, and a systemic response in

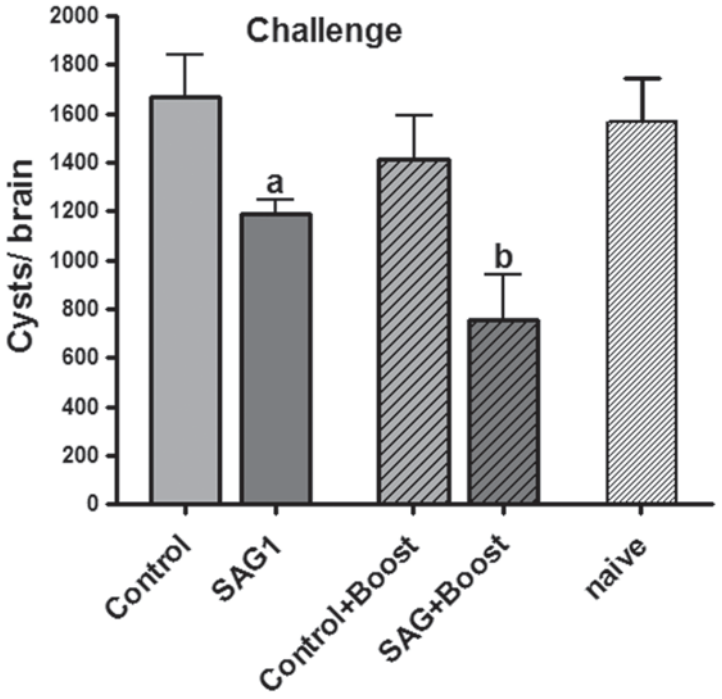

Fig. 11.2 Protection of C3H/HeN mice against *Toxoplasma* infection. Eight- to ten-week-old mice (8/group) were immunized on days 0, 14, 28, and 42 by subcutaneous injection. Two weeks after the last boost, mice were challenged by gavages with 20 cysts of the Me49 strain (LD50). Thirty days after the challenge, the number of brain cysts in mice was determined. Each bar represents the group mean ± S.E.M. $a = p < 0.05$: SAG vs. Control and naïve, and SAG1 + Boost vs. Control + Boost; $b = p < 0.01$: SAG1 + Boost vs. Control and SAG1 + Boost vs. naïve. *Control* mice vaccinated with pzp200-infiltrated leaf extracts, *SAG1* pKnS-infiltrated leaf extracts, *Control + Boost* pzp200-infiltrated leaf extracts + rSAG1 prime boost, *SAG1 + Boost* mice vaccine with pKnS-infiltrated leaf extracts plus rSAG1 prime boost. The results represent one of two similar experiments. Statistical analysis was performed by one-way analysis of variance (ANOVA) using Bonferroni's Multiple Comparison Test. (Taken from Laguía-Becher et al. 2010)

terms of GRA4-specific serum antibodies and secretion of IFN-γ, IL-4, and IL-10 by splenocytes (Figs. 11.6 and 11.7). This indicates that chlGRA4 displays a good potential for toxoplasmosis control by oral vaccination. Moreover, plant expression of GRA4 and SAG1 provides an excellent possibility for the development of a multicomponent vaccine against *T. gondii*.

Prospective View

Infectious diseases transmitted by parasites are important for their effects not only on human health but also on animal production. Parasitologists consider that the most efficient way to control parasitemias is through vaccination (Zucca and Savoia

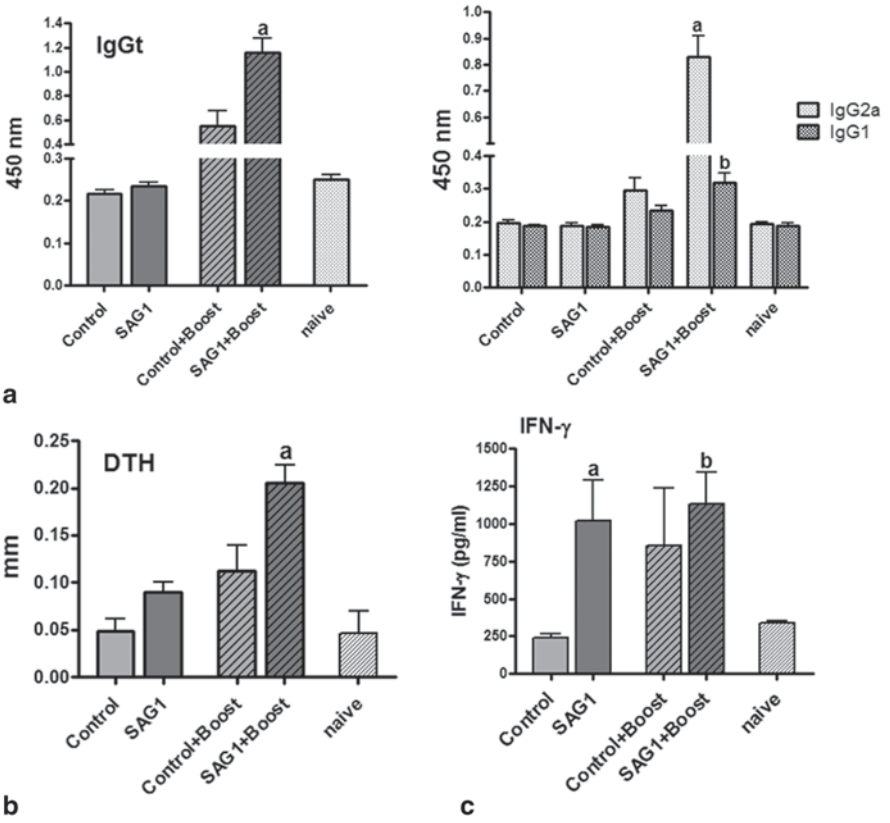

Fig. 11.3 Humoral and cellular response in C3H/HeN vaccinated mice. **a** Determination of specific anti-rSAG1 humoral response in C3H/HeN mice. Serum IgG profile in immunized mice determined by ELISA. *IgGt*: $a = p < 0.01$: SAG1 + Boost vs. Control + Boost; $b = p < 0.001$: SAG1 + Boost vs. Control, SAG1 and naïve; IgG2a: $a = p < 0.001$ SAG1 + Boost vs. Control, SAG1, Control + Boost and naïve; IgG1: $b = p < 0.01$ SAG1 + Boost vs. Control, SAG1 and naïve. Values for each serum sample were determined in duplicate. **b** Delayed-type hypersensitivity (*DTH*) to *Toxoplasma gondii* 48 h post intradermal injection in mice. $a = p < 0.05$: SAG1 + Boost vs. Control + Boost; $b = p < 0.01$: SAG1 + Boost vs. SAG1; $c = p < 0.001$: SAG1 + Boost vs. Control and naïve. **c** Cytokine production by splenocytes from vaccinated mice. Cells were harvested two weeks after the last immunization and cultured in the presence of rSAG1 (10 μg/ml). Supernatants were collected 72 h later and assessed for the production of IFN-γ by capture ELISA. $a = p < 0.05$: SAG1 and SAG1 + Boost vs. Control and naïve. *Control* mice vaccinated with pzp200-infiltrated leaf extracts emulsified with Freund's adjuvant, *SAG1* pKnS-infiltrated leaf extracts emulsified with Freund's adjuvant, *Control + Boost* pzp200-infiltrated leaf extracts + rSAG1 prime boost, *SAG1 + Boost* mice vaccine with pKnS-infiltrated leaf extracts plus rSAG1 prime boost. Results are expressed as the means value ± S.E.M and represent one of two similar experiments. Statistical analysis was performed by one-way analysis of variance (ANOVA) using Bonferroni's Multiple Comparison Test. (Taken from Laguía Becher et al. 2010)

2011). However, very few recombinant vaccines against parasitic diseases reach the market (Jacob et al. 2013). In fact, recombinant antigens produced in bacteria are sometimes not feasible due to the lack of posttranslational modifications or

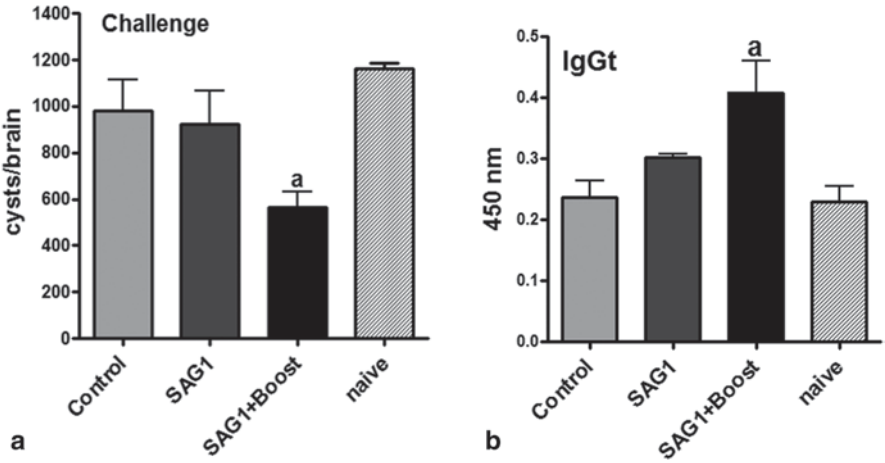

Fig. 11.4 Humoral response in orally immunized C57BL/6 (H-2d) mice and protection assay after a challenge with *T. gondii* cysts. **a** Cyst number per brain. $a = p < 0.05$: SAG1 + Boost vs. SAG1, Control and naïve. **b** Determination of specific anti-rSAG1 humoral response in C57BL/6 (H-2d) mice. Specific *IgGt* titers in sera from vaccinated mice were determined by ELISA. $a = p < 0.001$: SAG1 + Boost vs. SAG1, Control and naïve. *Control* mice vaccinated with pzp200-infiltrated leaf extracts, *SAG1* pKnS-infiltrated leaf extracts, *SAG1 + Boost* mice vaccinated with pKnS-infiltrated leaf extracts plus rSAG1 prime boost. Statistical analysis was performed by one-way analysis of variance (ANOVA) using the Bonferroni's Multiple Comparison Test. Results are expressed as the means value ± S.E.M and represent one of two similar experiments. Values for each serum sample were determined in duplicate. (Taken from Laguía Becher et al. 2010)

protein misfolding. On the other hand, the vaccine antigens expressed in eukaryotic cells (such as insects or yeast cell lines) may have posttranslational modifications that may affect their immunogenic properties, limiting the production of parasite antigens (Streatfield and Howard 2003). In this context, plants appear as an alternative for the production of functionally active parasite antigens (Clemente and Corigliano 2012). Edible-plant vaccines offer oral delivery, favoring the application and protection against pathogens by interacting with host mucosal surfaces via the induction of mucosal immunity. In this context, plant-based expression systems represent an interesting production platform for oral vaccine development due to their reduced manufacturing costs and high scalability (Hefferon 2010, 2012). However, despite these advantages, the heterologous expression of parasitic antigens in plants has been poorly explored. Among parasitic antigens expressed in plants, those derived from *Plasmodium* sp. (the cause of malaria) have also aroused great interest as *T. gondii* (Clemente and Corigliano 2012). In fact, it is noteworthy that parasitic antigens of veterinary interest have not been expressed in plants yet, being that vaccines for veterinary use tend to have fewer restrictions to commercialization than vaccines for human use (Jacob et al. 2013). Possibly one of the main constraints is that expression of parasite proteins in transgenic plants is difficult and time consuming, and that many of them are membrane proteins rich in hydrophobic regions, which further hamper their expression (Clemente et al. 2005).

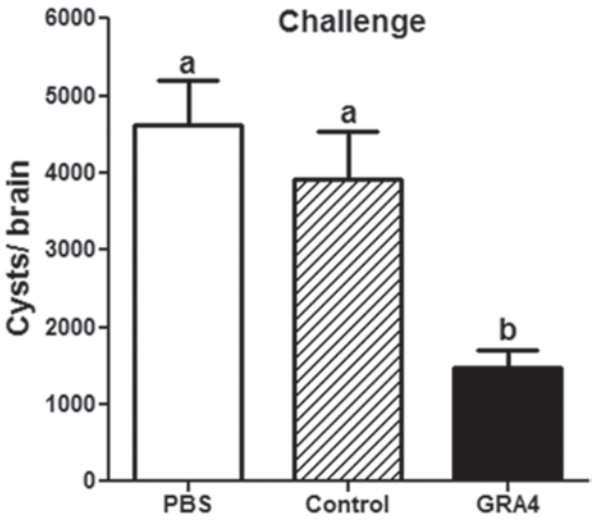

Fig. 11.5 GRA4 protection of C57BL/6(H-2d) mice against *Toxoplasma* infection. Eight- to ten-week-old mice (eight mice per group) were immunized on days 0, 7, 14, 21, and 28 by oral administration. Two weeks after the last boost, mice were challenged by gavages with 20 cysts of the Me49 strain (LD50). Thirty days after the challenge, the number of brain cysts in mice was determined. *Control* mice vaccinated with leaf extracts from wild-type plants, *GRA4* mice vaccinated with leaf extracts from chlGRA4 plants, *PBS* mice vaccinated with buffer PBS. Statistical analysis was performed by one-way analysis of variance (ANOVA) using the Bonferroni's Multiple Comparison Test. Results are expressed as the means ± S.E.M and represent one of two similar experiments. Different letters indicate statistically significant differences ($p < 0.001$ GRA4 vs. Control and PBS). (Taken from Del L Yácono et al. 2012)

In this context, methods should be optimized with regard to expression systems and vectors to make the production of recombinant proteins in transgenic plants more competitive. On the other hand, low production cost is a determining factor to use the plants and plant tissues for the synthesis of interesting proteins (Streatfield 2007). Given that the stability of foreign proteins can influence production yields and product concentrations, exploring new strategies that minimize the degradation of foreign protein may contribute to increasing the commercial value of production systems based on plants for the expression of parasite proteins in the development of oral vaccines.

Despite the lack of interest aroused by the proteins derived from parasites to be expressed in plants, the results shown in the present chapter are auspicious. The antigens of *T. gondii* in plants orally administered in low doses and without the use of adjuvants have been shown to be able to induce a protective immune response (Laguía-Becher et al. 2010; De L Yácono et al. 2012). In fact, the degree of protection observed is similar to that reported by other authors who assayed these proteins as recombinant proteins or DNA vaccines in combination with adjuvants (Martin et al. 2004; Sánchez et al. 2011). In addition, the protocol based on priming with plant-derived *T. gondii* SAG1 protein by oral administration and intradermal

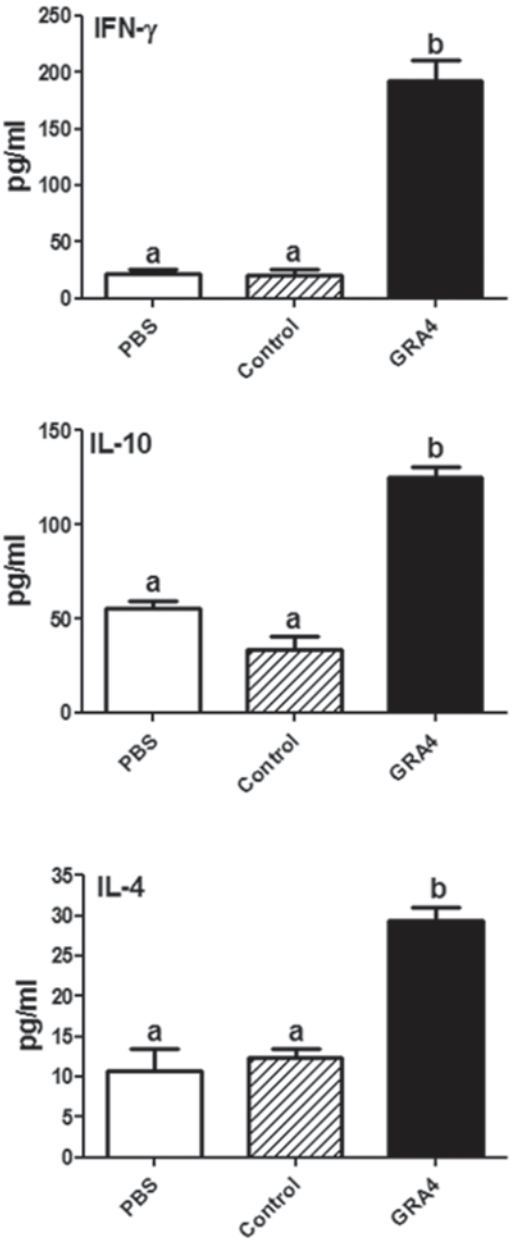

Fig. 11.6 C GRA4ytokine productions by spleen cells in orally vaccinated C57BL/6(H-2d) mice. Forty-five days after the immunization schedule was completed, spleen and lymph node cells were isolated from mice (five mice per group) and stimulated *in vitro* with excretory-secretory antigens (ESA) (10 μg/ml). Values for IFN-γ, IL-4, and IL-10 were measured at 72 h of culture. Results are mean cytokine concentrations in spleen cells from the five mice of each experimental group. *Control* mice vaccinated with leaf extracts from wild-type plants, *GRA4* mice vaccinated with leaf extracts from chlGRA4 plants, *PBS* mice vaccinated with buffer PBS. Results from one of two similar experiments are shown and are expressed as the mean ± S.E.M. Statistical analysis was performed by one-way analysis of variance (ANOVA) using the Bonferroni's Multiple Comparison Test. Different letters indicate statistically significant differences ($p < 0.001$ GRA4 vs. Control and PBS). (Taken from Del L Yácono et al. 2012)

boosting with rSAG1 expressed in bacteria elicited a protection (50%) similar to that obtained with chlGRA4 (59%; Laguía-Becher et al. 2010; De L Yácono et al. 2012). All these results suggest that the inclusion of exogenous adjuvants or an intradermal boost or GRA4–SAG1 mixed plant materials could be implemented

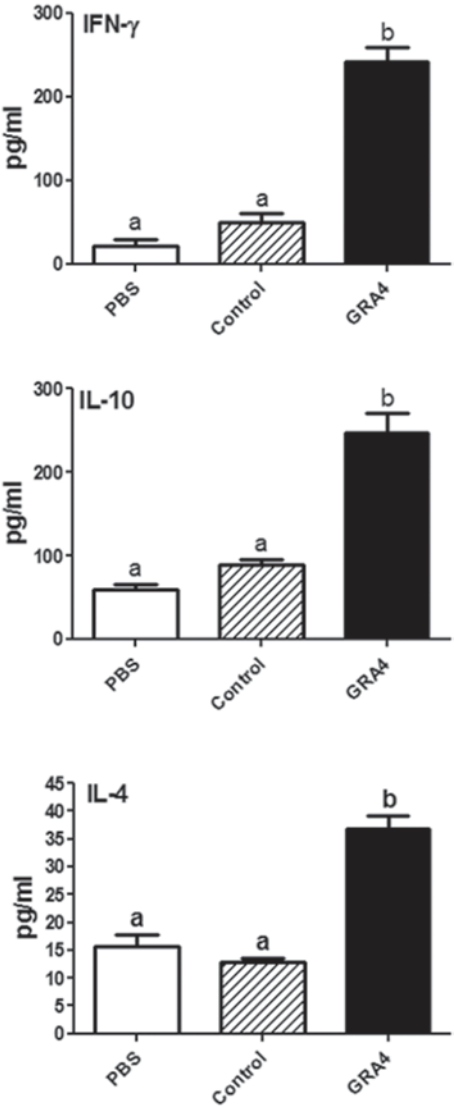

Fig. 11.7 Cytokine productions by lymph node cells in orally vaccinated C57BL/6(H-2d) mice. Forty-five days after the immunization schedule was completed, spleen and lymph node cells were isolated and stimulated in vitro with excretory-secretory antigens (ESA) (10 μg/ml). Values for IFN-γ, IL-4, and IL-10 were measured at 72 h of culture. Results are mean cytokine concentrations in lymph node cells from five mice per experimental group. *Control* mice vaccinated with leaf extracts from wild-type plants, *GRA4* mice vaccinated with leaf extracts from chlGRA4 plants, *PBS* mice vaccinated with buffer PBS. Results from one of two similar experiments are shown and are expressed as the mean ± S.E.M. Statistical analysis was performed by one-way analysis of variance (ANOVA) using the Bonferroni's Multiple Comparison Test. Different letters indicate statistically significant differences ($p < 0.001$ GRA4 vs. Control and PBS). (Taken from Del L Yácono et al. 2012)

to achieve a higher level of protection against *Toxoplasma* infection. Both GRA4 and SAG1 expressed in plants are good candidates for the development of a multi-antigenic vaccine against *Toxoplasma,* although it would be interesting to assess in future studies a wide range of leaf tissue doses and the maximization of the efficacy of the antigen presentation by using oral potent adjuvants.

In summary, the expression of *T. gondii* antigens in plants is a realistic strategy to develop an anti-*T. gondii* vaccine. The joint expression of SAG1 and GRA4 in plants may provide an excellent opportunity to explore the application of an oral vaccine against toxoplasmosis based on edible plant tissues.

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Chapter 12
Plant-Based Vaccines Against Pollen Allergy

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Introduction

Plant pollens are one of the most common causes of seasonal allergic diseases including allergic rhinitis, conjunctivitis, atopic dermatitis, and asthma. These occur as a consequence of fundamental allergenic mechanisms involving the induction of pollen-specific T helper type 2 (Th2) effector cells from naïve Th0 cells. Allergic diseases are characterized by allergen-specific immunoglobulin E (IgE) production and the activation of effector cells including eosinophils, mast cells, and basophils (Bousquet et al. 1998, 2009; Frew 2010). Immunological binding of allergen-specific IgE via Fc receptors (FcεRI) leads to mast cell and circulating basophil degranulation and release of the chemical mediators of inflammation. These events are regulated by Th2 cells, which preferentially produce interleukin (IL)-4, IL-5, and IL-13. Therefore, allergic diseases have been defined as the inadequate peripheral regulation of allergy-specific T cells.

Treatment strategies for these allergic diseases generally involve pharmacotherapies including antihistamines, leukotriene receptor antagonists, and corticosteroids (Holgate and Polosa 2008). Although these approaches reduce clinical symptoms by blocking the release of the critical mediators of allergic reactions or by inhibiting allergic inflammation, they are not curative and sometimes induce impaired performance as a result of side effects. Allergen-specific immunotherapy (allergen-SIT) is the only curative and antigen-specific method to treat allergic diseases by inducing immunological tolerance to the allergens responsible for the disease through multiple cellular and molecular mechanisms (Larche et al. 2006; Till et al. 2004). Such conventional allergen-SIT has been practiced for almost a century (Akdis and Akids 2007; Noon 1911). Success can be achieved by repeated subcutaneous injections of increasing doses of native allergen extracts over a period of at least 3–5 years in order to induce desensitization to the allergen. However, this treatment is sometimes

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accompanied by severe side effects, such as anaphylaxis, which is caused by the capture of the allergen together with a specific anti-allergen IgE on the surface of mast cells and circulating basophils.

It is highly desirable to develop safer, more effective, and convenient allergen-SITs to overcome these issues. To achieve this goal, tolerogens used for desensitization to allergens must be changed from crude allergen extracts to either modified hypoallergenic tolerogens with reduced IgE binding and enzymatic activity or the T cell epitope peptides required for the recognition of specific T cells, while maintaining T cell activity or immunogenicity. Second, the route of administration should be changed from systemic to mucosal (oral administration), resulting in increased convenience and relief from the pain of an injection.

Plants offer an ideal production platform for oral mucosal vaccines in terms of low production cost, no contamination by mammalian pathogens, high stability at ambient temperatures, and easy control of the production scale (Rybicki 2010; Yosibov and Rabindran 2008; Daniell et al. 2009). Production in seeds has already been demonstrated to give rise to a high yield of recombinant proteins based on established expression systems (Stoger et al. 2005; Lau and Sun 2009; Takaiwa 2011). The antigens that accumulate in seeds can be protected from degradation by the harsh environment of the gastrointestinal tract, and thereby provides a suitable delivery vehicle to gut-associated lymphoid tissue (GALT). Oral immune tolerance to allergens is expected to be induced in a manner similar to food proteins through the administration of a continuous dose of allergens in the diet. Seed-based allergy vaccines represent an innovative oral allergen-SIT for the control of allergenic diseases. We herein describe the feasibility of seed-based allergy vaccines against two representative pollen allergies.

Key Mechanisms Behind Allergen-SIT

Successful allergen-SIT by a conventional subcutaneous injection is associated with a decrease in allergen-specific IgE antibody production in the serum concomitant with the upregulation of IgG4 and IgA, which exhibit potential blocking activity by binding to the receptors of mast cells and basophils or competing with IgE for binding to the allergen (Meiler et al. 2008; Shamji et al. 2012). This altered IgE/IgG4 antibody balance also contributes to the reduction in allergen-induced IgE-mediated histamine release by mast cells and basophils and the inhibition of IgE-facilitated allergen presentation to T cells (James and Durham 2008), resulting in significant reductions in the numbers of infiltrating T cells, eosinophils, basophils, and neutrophils (Table 12.1).The induction of peripheral T cell tolerance is achieved by altering the balance between antigen-specific Th2- and Th1-type cells and/or by the induction of regulatory T cells (Tregs), leading to a reduction in inflammatory cell recruitment and activation and the suppression of mediator secretion from mast cells and basophils. The upregulation of Tregs from both naturally occurring thymus-derived forkhead box p3 (Fox $p3^+$) $CD4^+CD25^+$ regulatory T cells (nReg)

Table 12.1 Mechanisms of allergen-specific immunotherapy

Mode of action
1. Reduction in specific IgE levels
2. Induction of specific IgG4 and IgA as blocking antibodies
3. Reduction in basophil and mast cell activities
4. Shift to Th1 from Th2
5. T cell anergy or deletion of Th2 effector cells
6. Induction of regulatory T cells
7. Suppression of inflammatory DCs
Substances involved in action effects
1. Suppression of Th2 cell cytokine production (IL-4, IL-5, and IL-13)
2. Upregulation of Th1 cell cytokine production (IL12 and IFN-γ)
3. Upregulation of immunosuppressive cytokine IL-10 and TGF-β production
4. Suppression of the infiltration of T cells, eosinophils, basophils, and neutrophils
5. Suppression of the chemical mediators from mast cells and basophils
Effects induced by allergen-specific immunotherapy
1. Reduction in symptom scores (sneezing number and nasal itching)
2. Reduction in medication scores
3. Improvements in the quality of life (QOL)score
4. Long-term remission of symptoms (prevention of progression)
5. Prevention of the onset of new sensitizations

and peripheral inducible Tregs (iTregs) such as type 1 Tregs (Treg 1), Th3 cells, and mucosally induced Fox $p3^+CD4^+CD25^+$ cells ($Foxp3^+$iTreg), suppresses the development of allergic diseases via several immune mechanisms including the suppression of dendritic cells (DCs), Th cells, mast cells, eosinophils, and basophils in the skin, nose, eye, and bronchial mucosa (Jutel and Akdis 2011; Ozdemir et al. 2009; Palomares et al.2010).

The specific inhibition of T cell proliferation and allergenic inflammation is dependent on the suppressive cytokines IL-10 and TGF-β released by different populations of Tregs and DCs (Jutel et al. 2003). IL-10 is produced by Treg1, Fox $p3^+$ iTregs, $CD8^+$ Treg, γδ T cells, and DCs, whereas transforming growth factor-β (TGF-β) is mainly produced by Th3 cells and Fox $p3^+$ nReg. IL-10 directly acts on $CD4^+$ T cells and downregulates the production of IL-2 and IFN-γ by Th1 cells and of IL-4, IL-5, and IL-13 by Th2 cells. The production of allergen-specific IgE and expression of the IgE receptor are suppressed by IL-10, whereas it induces a class switch toward IgG4 production. Moreover, allergic inflammation in peripheral tissue is also suppressed by decreasing proinflammatory cytokine release from mast cells and depressing eosinophil activities (Ozdemir et al. 2009; Palomares et al. 2010). On the other hand, TGF-β is involved in the conversion of naïve T cells in Tregs, inhibition of the proliferation of T cells and B cells, downregulation of effector cytokine production, and suppression of macrophages, DCs, and natural killer cells (Yoshimura et al. 2010). Moreover, it has also been implicated in the induction of class switching to IgA.

Therefore, the basic principle of allergen-SIT is to induce immune tolerance to the allergens causing the disease through multiple cellular and molecular mechanisms, leading to a reduction in inflammatory cell recruitment and activation and also mediator secretion from mast cells and basophils. The induction of a tolerant state in peripheral T cells represents an essential step in allergen-SIT.

Advantage of Seed-Based Allergy Vaccines as a Delivery System to GALT

When crude antigen extracts used in subcutaneous injections are orally administrated, they are generally subjected to proteolysis in the gastrointestinal tract before they arrive at immune cells in GALT, resulting in little significant efficacy. Thus, to achieve the same level of efficacy as that of a subcutaneous injection, oral administration requires doses that are hundreds of times higher. This limitation makes it impossible to establish allergen-SIT for allergic diseases via oral administration (Polovic and Velickovic 2008).

However, irrespective of the markedly lower concentrations than those required from naked allergen proteins, plant-based antigens are expected to be effectively delivered to mucosal immune cells in GALT through protection from harsh conditions in the gastrointestinal tract, and immune tolerance can be easily induced when vaccines are produced in plant cells and directly administered through the oral route (Streatfield 2006; Pelosi et al. 2012; Paul and Ma 2010). This may be explained by the bioencapsulation of antigens with double barriers, consisted of the cell wall and intracellular compartments such as the protein body (PB). Furthermore, because cells containing antigens are filled with starch or lipid, the antigen may be gradually released to the gastrointestinal tract. Thus, when antigen presentation to antigen-presenting cells (APC) is prolonged under these conditions, the efficiency of immune responses (tolerance) is improved.

When digestibility and immune tolerance-inducing capacity were compared between endoplasmic reticulum (ER)-derived PB (PB-I)-containing and protein-storage vacuole (PSV; PB-II)-containing antigens, the resistance of the ER-derived PB in cereal grain to gastrointestinal digestion enzymes was previously shown to be stronger than that of the PSV (Takagi et al. 2010). This physical difference may be related to the polymerized or aggregated formulation of the antigen observed in ER-derived PBs, which are formed by disulfide bonds through cys-rich prolamins. Interestingly, several prolamins with different physical properties in cereal grains have been shown to be tightly packaged in a specific arrangement in ER-derived PBs during seed maturation (Lending and Larkins 1989; Saito et al. 2012; Takaiwa 2013a). Taken together, the resistance of antigens to proteolysis against digestive enzymes is associated with the efficacy of edible vaccines. Physical bioencapsulation within plant cells provides an effective delivery system for an oral vaccine.

Induction Mechanisms of Oral Immune Tolerance

Immune tolerance levels through the oral route have been reported to vary depending on the dose, frequency, or formulation of the administrated antigen (Mayer and Shao 2004; Burks et al. 2008; Weiner et al. 2011). A soluble antigen is known to be more tolerogenic than a particulate one, although the latter is more resistant to the harsh conditions in the gastrointestinal tract than the former. However, protection from proteolysis by digestive enzymes has a crucial effect on the efficacy of antigens. Furthermore, oral administration can induce both mucosal and systemic immunity, whereas parenteral delivery cannot. Thus, the selection of an oral route is rational as an administration method because immune tolerance is more prone to be induced than by immune stimulation.

The high-dose administration of an antigen (100–500 mg) was shown to result in the deletion or anergy of lymphocytes (T cells) (Mayer and Shao 2004). Fas (CD95)-dependent apoptosis was responsible for the deletion of effector T cells (Marth et al. 1998). Allergen-specific T cell anergy occurs when incomplete activation signals are sent through T cell receptor (TCR) interactions between factors such as B7-1 (CD80) or B7-2 (CD86) (B7 family) on APC with CD28 (B28 family) on T cells or when a lack of costimulatory molecules occurs during this activity (Appeman and Boussiotis 2003). In addition to the original B7/CD28 family members, several B7 family members such as inducible costimulator ligand (ICOSL (B7-H2)), PDL-1 (B7-H1), PDL-2 (B7-DC), and B7-H3 have been identified to date. Several CD28 families including B7-1 (CD80), PD-1, cytotoxic T lymphocyte antigen-4, and inducible costimulator (ICOS) have also been identified (Greenwald et al. 2005; Keir et al. 2008). Downregulation of the costimulation molecule CD80/CD86 on APC was observed with an increase in programmed death ligand (PDL-1) as an inhibitory costimulator through the induction of anergy in the process of oral immune tolerance (Piconi et al. 2010). High PDL-1 and PDL-2 expression levels in DCs in the gut have been implicated in oral tolerance through the induction of antigen-specific Tregs. The ICOSL was also shown to be involved in the induction of Tregs. Thus, interactions involving costimulatory molecules play a key role in the regulation of T cell activation and tolerance.

In contrast, the repeated administration of a low-dose antigen (1–5 mg) was shown to be mediated by active immune suppression through the induction of Tregs, such as $CD4^+$ iTregs, Fox $p3^+$ iTregs, Th3 cells, Tr1 cells, Fox $p3^+$ $CD4^+$ $CD25^+$ nTregs, $CD8^+$ T cells, and γδ T cells (Weiner et al. 2011). These Tregs express anti-inflammatory IL-10 or TGF-β cytokines as well as CTRA-4 as the costimulation factor. The CTLA-4 released from Fox $p3^+$ $CD4^+$ $CD25^+$ is known to bind to CD80 and CD86 on APCs with a higher affinity than CD28 and to counteract the activation delivered by the TCR. $Foxp3^+$ $CD4^+$ $CD25^+$ nTregs in particular involve the expression of cell-surface-bound TGF-β, which can act as a cognate suppressive factor. IL-10-producing immature DCs ($CD11b^+$) and $CD103^+$ DC in the gut have been shown to contribute to immune tolerance through the induction of Tr1 and $Foxp3^+$ iTregs, respectively (Lafaille and Lafaille 2009; Belkaid and Oldenhave

2008). Lamina propria (LP) macrophages can efficiently induce Foxp3$^+$ T cells in the presence of TGF-β. The tolerogenic capacity of DCs depends on the maturation stage. Immature or partially mature DCs have the ability to induce peripheral tolerance through the generation of Tregs, whereas fully mature DCs prime naïve T cells to different effector Th cells. It is generally accepted that myeloid DC and plasmacytoid DC (pDC) are different functional subsets. The pDCs play an essential role in the prevention of allergy sensitization by inducing IL-10-producing Tregs.

The increased levels of IL-10 and TGF-β potently suppress allergen-specific IgE production from B cells by inducing a class switch toward the non-inflammatory isotype IgG4 and mucosal IgA production, respectively. Tregs are able to form aggregates around DCs, which inhibit their maturation and also downregulate their costimulatory molecules. In addition, Tregs directly or indirectly suppress the effector cells of allergic inflammation such as mast cells, basophils, and eosinophils as well as Th2, by producing the regulatory cytokines TGF-β and IL-10 (Ozdemir et al. 2009; Palomares et al. 2010; Jutel et al. 2003). The suppression of mast cells (FcεRI-dependent mast cell degranulation) by Tregs was previously shown to be mediated through cell–cell direct contact involving OX40–OX40 ligand interactions (Gri et al. 2008). In conclusion, peripheral tolerance to allergens is controlled by multiple active suppression mechanisms. (Table 12.2)

The differentiation of IL-10-producing Treg 1 is induced by IL-6, IL-27, and TGF-β, whereas IL-6, IL-23, and TGF-β are responsible for the full differentiation of Th17 implicated in autoimmunity and pathogen attacks (Bettelli et al. 2006; Mucida et al. 2007; Awasthi et al. 2007; McGeachy et al. 2007). Both IL-6 and IL-27 are produced by DCs and macrophages. The conversion of Fosp3$^+$ iTregs from naïve CD4$^+$ T cells is also induced by TGF-β and retinoic acid (RA), which are produced in DCs (CD103$^+$ DC) with retinal dehydrogenase activity in the small intestine and mesenteric lymphoid nodes (MLN) of the gut (Mucida et al. 2007). Therefore, DCs from the small intestine and MLN are efficient in the peripheral conversion of Tregs. RA suppresses differentiation from naïve T cells to Th17, while IL-6 inhibits TGF-β-induced Foxp3$^+$ iTreg induction, resulting in the differentiation of Th17 (Mucida et al. 2007; McGeachy et al. 2007). Th3 cells contribute to the induction of Fox p3$^+$ iTregs through the production of TGF-β. TGF-β also induces the Runt-related transcription factors RUNX1 and RUNX3 that bind to the *FOXP3* promoter, which participates in the development and function of Tregs. Inactivation of the RUNX cofactors leads to a decreased number of iTregs.

Uptake of Antigens and Immune Reactions in GALT

When antigens capsulated in the aggregate formulation such as PBs were orally administered, they were demonstrated to cross the intestinal epithelial cell barrier in several ways (Mayer and Shao 2004; Burks et al. 2008). Particulates of <10 μm such as PBs (usually 1–2 μm) are principally taken up by M cells in the follicle-associated epithelium (FAE) covering Peyer's patches (PPs) as part of

Table 12.2 Regulatory T cells and dendritic cells involved in immune tolerance and their effects

Regulatory T cells	*Secreted cytokines*
Thymus-derived natural Fox p3$^+$ T cell (nFox p3)	TGF-β
Inducible Foxp3$^+$ T cell (iFox p3)	IL-10, TGF-β
Treg1(Tr1)	IL-10
Th3	TGF-β
CD8$^+$ Treg	IL-10
Effect of regulatory T cells	
1. Suppression of effector Th2 cells	
2. Suppression of effector Th1 cells	
3. Suppression of effector Th17 cells	
4. Anergy of T cells	
5. Suppression of T cell migration to tissues	
6. Direct and indirect suppressive effects on mast cells, basophils, and eosinophils	
7. Suppression of inflammatory DC and the induction of IL-10-producing DCs	
8. Induction of IgG4 by B cell class switch (IL-10)	
9. Induction of IgA by B cell class switch (TGF-β)	
Dendritic cells (DCs)	*Induced effector T cells*
CD103$^+$ DC (retinoic acid (RA)+TGF-β)	iFoxp3$^+$Treg.
DC (IL-10+RA)	Tr1
DC (TGF-β+IL-6+IL-27)	Tr1
DC (TGF-β+IL-6)	Th17
CD11c$^+$ DC (TGF-β)	Th3
CD11b$^+$ DC (IL-10, IL-27)	Tr1
Plasmacytoid DC	Tr1
CD11b$^+$(TGF-b$^+$IL-6$^+$IL10)	Th2
IDO$^+$CD11c$^+$	iFoxp3$^+$Treg

GALT in the small intestine and are then presented to APCs such as DCs in adjacent mucosal T cell areas. M cells act as a portal for the uptake of antigens from the intestinal lumen that are transferred into DCs in the PPs, which are composed of 5–10% of cells in FAE. As examples of other routes, antigens up to the size of a few micrometers or nanometers are processed and presented by intestinal epithelial cells. CD103$^+$ DCs themselves are capable of extending their dendrites through the epithelium (tight junctions) in the LP into the gut lumen to catch antigens without disrupting the tight junctions and function as a conduit for the delivery of antigens. Subsequently, antigens sampled by CD103$^+$ DCs are carried to the local MLN and are then presented to specific T cells, which indicates that MLN plays a central role in the acquisition of oral tolerance (Weiner 2011; Tsuji and Kosaka 2008). Moreover, liver pDCs can also contribute to the acquisition of tolerance against oral antigens because the liver stores up to 80% of total body retinol.

GALT contains an organized macro-architecture of B and T lymphocyte zones that respond to the antigens presented by DCs, which can induce memory B and T cells (Tsuji and Kosaka 2008; Neutra and Kozlowski 2006). B cells are a major

component of PP cells, comprising more than 70%, and are preferentially located in the follicle region. Formation of the germinal center occurs in the PPs, in which class switching of B cells from IgM to IgA is achieved. T cells account for approximately 20% of PP cells and are mainly located in the FAE region that mainly contains naïve T cells (Kunisawa et al. 2012). Differentiation into IFN-γ-producing Th1, IL-4-producing Th2, or IL-10-producing Fox $p3^+$ Tregs is induced depending on the presentation of various types of DCs. There are at least three subsets of DCs with distinct tissue distribution in PPs (Milling et al. 2010). $CD11b^+$ ($CD11b^+CD8\alpha^-$) myeloid DCs are present in subepithelial domain (SED) regions, $CD8^+$ ($CD11b^-CD8\alpha^+$) lymphoid DCs in the T cell-rich interfollicular region (IFR), and double-negative $CD4^-CD8\alpha^-$DCs in both SED and IFRs (Neutra and Kozlowski 2006). DCs in the latter two regions produce IL-12 and induce IFN-γ-producing Th1 cells, which are responsible for pathogen clearance. The production of IL-10 and IL-27 by $CD11b^+$ cells (myeloid DCs) plays a critical role in oral tolerance by inhibiting the differentiation of naïve T cells to Th 17 cells as well as enhancing IL-10 production by Tregs, since $CD11b^+$ cell-deficient animals have a defect in oral tolerance. In contrast, $CD11c^+$ mucosal DCs preferentially produce anti-inflammatory cytokines (Fig. 12.1).

Another important component of GALT, which serves to regulate intestinal homeostasis, is intraepithelial lymphocytes (IELs), which constitute 10–20% of epithelial cells. Approximately 30–40% of all peripheral T cells are present in IELs. The lack of IELs results in the loss of oral immune tolerance, which indicates their significant participation in gut oral immune tolerance. The majority of IELs contain $CD8^+$ T cells with regulatory activity, which express αβ or γδ TCRs Kunisawa et al. 2007). $CD8^+$ Tregs are involved in oral tolerance by suppressing Th1 and Th17 responses. γδ T cells are important mediators of mucosal tolerance.

Benefits of Hypoallergenic Tolerogens

Many advantages are associated with replacing the natural allergen extracts used to induce immune tolerance (desensitization) with recombinant proteins having reduced allergenicity (low IgE binding activity), because the former can bind to specific IgE on mast cells and basophils, leading to anaphylactic side effects (Valenta et al. 2010). Therefore, new approaches to SIT using modified hypoallergenic antigen derivatives (so-called hypoallergens) are required in order to increase the safety of immunotherapy by reducing the risks of anaphylactic reactions relative to the corresponding natural allergen (Focke et al. 2010). Binding of an allergen to the specific IgE was previously shown to be determined by a continuous stretch of amino acids (B cell epitope) or conformational structures. Thus, in order to develop such ideally hypoallergenic tolerogens as immune modulators, deletion, site-directed mutagenesis, fragmentation, oligomeric formation, or molecular shuffling have all been tested (Valenta et al. 2011; Linhart and Valenta 2005; Cromwell et al. 2011). Recombinant hypoallergenic allergen derivatives exhibit reduced IgE

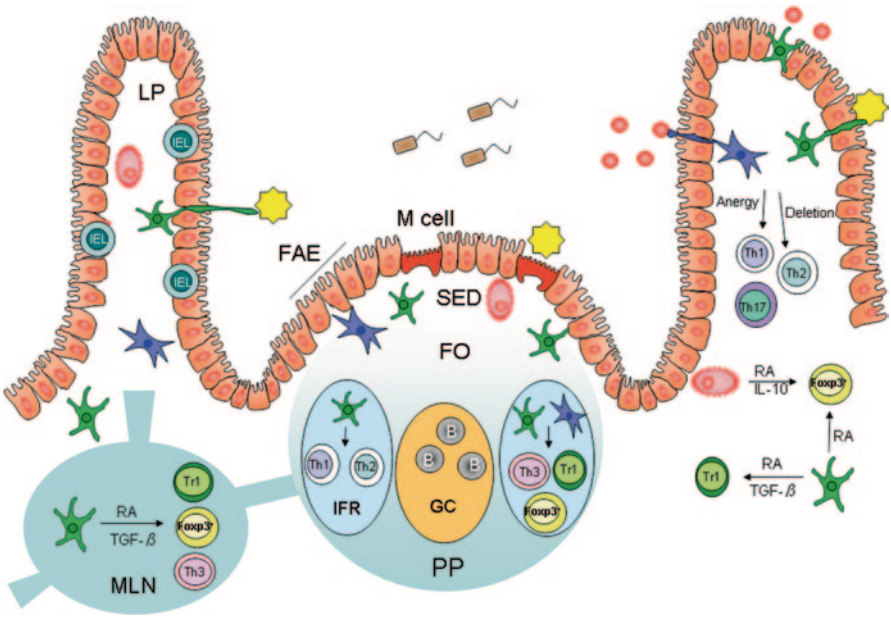

Fig. 12.1 Mechanisms of oral tolerance induction. Orally delivered antigens can be captured by antigen presentation cells (*APCs*) such as DCs and macrophages in the GALT. DCs and macrophages in the subepithelial dome of peyer's patches (*PPs*) and in the lamina propria (*LP*) of the intestine take up fed antigens via M cell overlying PPs. LP DCs can directly sample luminal antigens by extending their dendrites between IECs. DCs can present the antigens to T cells or after migration to the mesenteric lymph node (*MLN*), an attending lymph node in the intestinal mucosa. A number of different types of Tregs ($Foxp3^+$iTregs, nTregs, Tr1 cells, Th3 cells, $CD8^+$ Tregs, and γδT cells) are induced or expanded in the gut and are involved in oral tolerance. *FO* follicle, *FAE* follicle-associated epithelium, *GALT* gut-associated lymphoid tissue, *GC* germinal center, *IEL* intraepithelial lymphocytes, *IFR* intrafollicular region, *LP* lamina propria, *MLN* mesenteric lymph node, *PP* peyer's patch, *SED* subepithelial domain

reactivity; hence, they do not induce IgE-mediated side effects when administered to allergic patients.

Vaccinations with these genetically modified recombinant allergen derivatives induced the production of protective IgG4 antibodies against specific IgE, which was accompanied by improvements in clinical symptoms and skin sensitivity. Furthermore, hybrid molecules consisting of multiple allergens have been developed for several important allergen sources such as birch pollen allergens and timothy glass pollen allergens. Vaccinations with hybrid molecules composed of five timothy grass pollen allergens significantly improved grass pollen-induced immediate-type allergic symptoms and resulted in the reduced consumption of medication (Linhart et al. 2002).

On the other hand, peptide immunotherapy using dominant T cell epitopes derived from allergens offers a safe and ideal treatment for controlling allergic diseases because regions involved in allergenicity can be completely excised while

ensuring the retention of immunogenicity (Larché 2007; Moldaver and Larche´ 2011). It should be noted that designing effective peptide vaccines for human immunization is complicated by the polymorphism of MHC and antigenic complexity of allergens. Hybrid peptides composed of multiple major T cell epitopes from bee venom (Api ml) and cat allergens (Fel d 1) have been created to compensate for genetic viability. Hybrid peptides can also be engineered to preserve most allergen-specific T cell epitopes derived from a few molecules by linking several T cell epitopes.

Production of Tolerogens in Plants

To produce the allergy vaccines (tolerogens) required to induce immune tolerance by oral administration in plants, it is important to increase their production levels as much as possible. Enhancing strategies for vaccine levels include strong tissue-specific promoters, codon optimization, translation fusion, targeting expression to specific tissues or subcellular localization, and introgression of transgenes into the germplasm more suitable for high-level expression (Streatfield 2007; Sharma and Sharma 2009; Kawakatsu and Takaiwa 2010).

Several parameters that determine transcription, translation, and posttranslational modifications have to be optimized to boost accumulation levels. Expression levels are primarily determined by transcription levels; therefore, it is critical to employ strong tissue-specific promoters to enable higher levels of gene expression in targeted deposition tissue. Seed-specific promoters are desirable for the high-level accumulation of recombinant proteins because the constitutive expression of recombinant proteins by CaMV 35S, ubiquitin, or actin promoters has sometimes detrimental or vital effects on vegetative growth (Lau and Sun 2009; Sharma and Sharma 2009).

The endosperm of cereal grain accounts for more than 80% of total seed weight and is a specialized storage organ for starch and proteins, whereas the embryo or cotyledon is a storage organ of dicot seeds. Rice major seed storage protein (SSP) glutelin (*GluB-1, GluB-2, GluA-2*), 26-kDa globulin, or 10-kDa and 16-kDa prolamin promoters have been used as strong endosperm-specific promoters for the expression of recombinant proteins in transgenic rice seed (Qu and Takaiwa 2004). Foreign products highly accumulated as detectable proteins in the CBB-stained sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels of total SSPs, accounting for up to 12% of total seed proteins (10 mg/g dry seeds). In maize and barley seeds, γ-zein, globulin, and hordein promoters have been used for these transgenic plants. When expressed in dicot seeds, soybean glycinin and β-conglycinin, common bean β-phaseolin and arcelin-5 promoters, pea legumin (legA), and broad bean unknown seed protein (USP) promoters have been employed and resulted in high yields of target recombinant proteins, which reached a level 36.5% of total seed proteins at the maximum yield (Lau and Sun 2009; Sharma and Sharma 2009).

To improve accumulation levels, it is critically important to optimize codons in the coding sequence by exchanging rare codons with those frequently used in highly expressed plant genes taking the difference in codon bias into consideration, and further accumulation can be achieved by eliminating the A/U-rich mRNA-destabilizing motif, AUUUA motif, and the polyadenylation signal, AAUAAA motif. Codon optimization resulted in a 5–100-fold enrichment of the accumulation yield over that of the original one (Gustafsson et al. 2004).

Furthermore, the 5′ and 3′ untranslated regions (UTRs), which are involved in translation efficiency or mRNA stability, must also be considered. The complete 5′ and 3′ UTRs derived from SSPs are usually attached to the coding sequence of target recombinant proteins (Sharma and Sharma 2009). Use of the 3′ UTR derived from the SSP gene terminated more specifically at a few sites than the noparine synthase (Nos) terminator, leading to improvements in accumulation level by stabilizing transcripts.

Targeting to Intracellular Compartments

The trafficking process and intracellular localization site of expressed recombinant proteins have a crucial influence on folding, assembly, and posttranslational modifications such as glycosylation (Benchabane et al. 2008). Therefore, it is important to deposit recombinant proteins into the suitable intracellular site, which allows stable and ample storage. The accumulation capacity may be affected by the individual intracellular compartment determining the deposition space (Khan et al. 2012). For example, the endosperm cells of cereal seeds are dominated by starch granules and PBs, whereas dicotyledonous legumes or rape seed cells are filled with protein and oil bodies.

The ER is an entry point for the secretory pathway and secretory proteins are correctly folded in the ER lumen before sorting. Attachment of the signal peptide to the N-terminus of the recombinant protein is essential for the expression of recombinant proteins as secretory proteins. When pharmaceutical proteins such as vaccines and antibodies are produced in plants, targeting to the secretory pathway generally improves accumulation levels as a result of the higher folding ability of ER-resident chaperons, such as binding proteins (BiP) and protein disulfide isomerase (PDI), or the low proteolytic activity in the ER lumen. Secretory proteins are subject to assessment by protein quality control in the ER lumen prior to trafficking to the destination site via the endomembrane system (Vitale and Boston 2008). If they cannot be properly folded or assembled, unfolded or misfolded proteins are finally degraded by the ER-associated degradation (ERAD) system. In the case of plant-based allergy vaccines, high amounts of destructed hypoallergenic allergens have to be produced for efficacy; therefore, there is a high possibility that ER stress or the unfolded protein response may be highly induced by the accumulation of unfolded or misfolded proteins. At least two signaling pathways have been implicated in the ER stress response in plants, the orthologs of IRE1/XBP1 and ATF6, whereas

the plant counterpart of PERK has not been identified (Howell 2013). Many genes coding for chaperons and folding enzymes are upregulated through these ER stress signaling pathways as a result of the unfolded protein response. Therefore, enriching the ER quality control capacity (protein folding capacity) is necessary to control the ER stress signaling pathway determining chaperon levels to enhance the accumulation levels of foreign proteins.

As a unique strategy, higher levels of recombinant proteins could be obtained by their expression in specific tissues and targeting to intracellular compartments such as PB or PSV through the simultaneous suppression of endogenous seed proteins by an RNAi-based approach because the deposition space is enlarged by the vacancy of endogenous seed proteins and compensatory mechanisms work to maintain total nitrogen and sulfur levels in seed proteins (Takaiwa 2013b; Wu et al. 2012). This improvement in the recombinant protein yield was also observed with the expression of recombinant proteins in a host (germplasm) deficient or suppressed in some seed proteins. This has been attributed to the rebalancing or compensatory effect of proteins to maintain the same nitrogen levels in seeds and to lessen competition for deposition in a limited space between endogenous and foreign proteins.

When proteins are transported as secretory proteins into the ER, high mannose type glycans are added to accessible asparagines in the amino acid sequence context Asn-Xaa-Ser/Thr and more complex glycans are built on the mannose framework in the Golgi apparatus, including the addition of β-1,2-xylose and α-1,3-fucose at particular sites in the glycan (Gomrd et al.2010). Plants can be genetically modified to produce glycans that are more similar to those found in animals by deleting or suppressing plant-specific glycosylating enzymes genes such as α-1,3 fucosylransferase and β-1,2 xyltransferase through mutation or homologous recombination (gene knockout), suppressing their expression by RNAi (knock down), or by predominantly targeting the ER lumen through retrieval from the Golgi apparatus by ligating the C-terminal KDEL ER retention signal.

Recombinant proteins can currently be artificially targeted to various subcellular compartments such as ER lumen, Golgi complex, PB, PSV, or apoplast as well as chloroplast and mitochondria by fusing to the targeting signal peptide such as the transit peptide, vacuolar sorting signals (VSS), or ER retention signal (Benchabane et al. 2008; Vitale and Hinz 2005). Proteins with no N-terminal signal peptide result in targeting to the cytoplasm. Ligation of the signal peptide and the KDEL/HDEL ER retention signal at the N- and C-termini of recombinant proteins generally enables accumulation in the ER lumen by retrieval from the Golgi apparatus, resulting in more than a 10-fold enhancement in yield over that without the ER retention signal.

A fusion strategy further ensures transportation to the expected intracellular site as well as the enrichment and stability of products. Hybrid proteins with seed proteins, zera (proline rich N-terminal domain of the maize γ-zein), elastin, ubiquitin, and immunoglobulins (HIV-1 p24-immunoglobulin fusion) have been reported to be useful in enhancing stability and folding (Conley et al. 2011; Torrent et al. 2009; Floss et al. 2009; Obregon et al. 2006; Hondred et al. 1999). Fusion with the N-terminal portion of γ-zein or elastin-like polypeptide, the multiple repeats of the

elastin motif (VPGXG), resulted in PB formation even in vegetative tissue and enhanced production.

As an alternative, fusion with native SSP, in which foreign proteins are inserted in a highly variable region such as the C-terminal region of the rice glutelin acidic subunit and 26-kD globulin, stably accumulated as part of the seed protein and resulted in targeting to inherent PSVs, allowing enrichment of the recombinant protein in the PB (Wakasa et al. 2006). On the other hand, when recombinant protein was fused to the C-terminus of various prolamins, they were also stably deposited to ER-derived PBs. Therefore, recombinant proteins can be targeted to the desired compartment according to the properties of the fusion partner.

Rice Seed-Based Birch Pollen Allergen Against Birch Pollen Allergy

Birch pollen is widely distributed in Europe, North America, Russia, and northern Japan. The major pollen allergens of Fagales trees belonging to the same order as birch are known as the Bet v 1 family because of the strong IgE cross-reactivity between Bet v 1, a major birch pollen allergen, and the homologous allergens observed in alder (Aln g 1), hazelnut (Cor a 1), hornbeam (Car b 1), and oak (Que a 1) pollen (Weber 2006). Bet v 1 is a glycoprotein, with a molecular weight of approximately 20 kDa that belongs to the PR10 protein group. More than 90% of birch pollen allergy patients are sensitized to Bet v 1-specific IgE. The Bet v 1 allergy is also known to cause oral allergy syndrome, as the following food allergens are cross-reactive with Bet v 1-specific IgE: apple Mal d 1, soybean Gly m 4, carrot Dau c 1, and peanut Aha h 8 (Vieths et al. 2002).

The creation of versatile recombinant hypoallergenic allergens retaining all T cell epitopes and immunogenicity should provide an ideal tolerogen for allergen-SIT. Hypoallergenic Bet v 1 derivatives against multiple Fagales pollen allergens were previously generated through in vitro random recombination by means of DNA shuffling (Wallner et al. 2007). Importantly, TPC7 and TPC9 not only exhibited lower allergenicity than native Bet v 1, as determined by IgE reactivity and basophil activation assays, but TPC7 also showed a 20-fold reduction in IgE binding capacity and an 8- to 10-fold reduction in basophil degradation activity relative to wild-type Bet v 1. Therefore, this TPC7/TPC9 chimera should be a suitable tolerogen for SIT not only against birch pollen allergy but also against allergies caused by other cross-reactive tree pollen.

A codon-optimized TPC7 synthetic gene, in which the GluB-1 signal peptide and the KDEL ER retention signal were fused to the N- and C-termini, respectively, was then expressed in stable transgenic rice seeds under the control of the endosperm-specific 2.3-kb glutelin *GluB-1* promoter (Wang et al. 2013). Recombinant TPC7 was produced as a glycoprotein with high mannose-type N-glycan, but without β-1,2-xylose or α-1,3-fucose, which suggests that TPC7 is retained in the ER. TPC7 strongly accumulated in the endosperm tissue to approximately 200 μg/grain, lead-

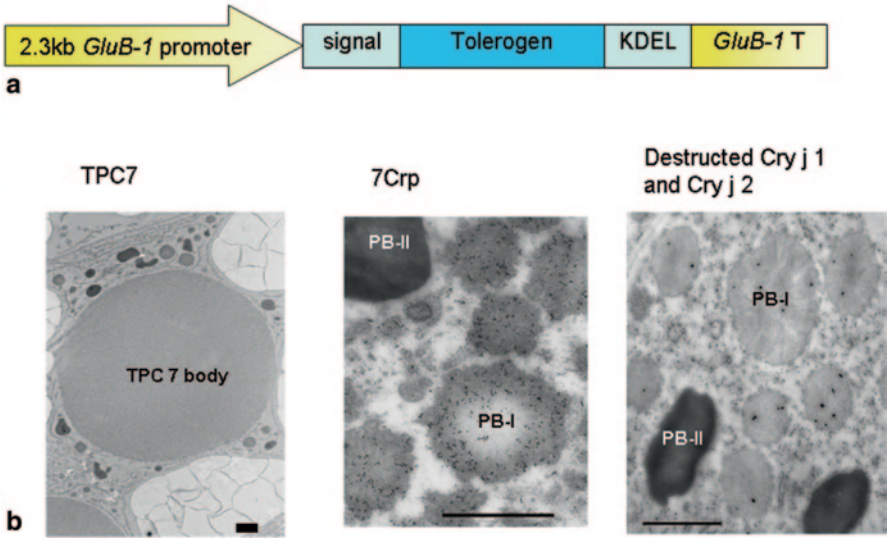

Fig. 12.2 Expression system of various tolerogens in transgenic rice seeds and the intracellular structure of endosperm cells containing PBs. **a** General expression system used for the production of tolerogens (pollen allergen derivatives) in transgenic rice seeds. **b** Intracellular structure of developing endosperm cells containing PBs depositing tolerogens in transgenic seeds. The intracellular localization of recombinant proteins was analyzed using immunoelectron microscopy. *PB-I* protein body I, *PB-II* protein body II, Bar 1 μm

ing to the suppression of cysteine-poor 13-kDa prolamin and 26-kDa globulin production. Interestingly, when the intracellular location of TPC7 was examined, it was found to be deposited into a very large organelle, more than 20 μm in diameter and is referred to as the TPC7 body, and was distinct from PB-I and PB-II (Fig. 12.2). The TPC7 body is predominantly filled with TPC7 proteins and interactions rarely occur with endogenous SSPs. Transgenic rice seed with high amounts of TPC7 may be a potential candidate as an oral tolerogen against birch pollen allergy.

Rice Seed-Based Allergy Vaccine Against Japanese Cedar Pollen Allergy

Japanese cedar pollen allergy is an important public health problem in Japan. Approximately 30% of the population is currently afflicted with this pollinosis between February and April each year (Okamoto et al. 2009). People with circulating specific IgE against cedar pollen allergens (potential patients) account for up to 60% of the general population. The main allergens causing this pollinosis are Cry j 1 and Cry j 2, the major T cell epitopes of which have been well characterized. Cry j 1 has pectate lyase activity and is specifically localized in the pollen cell wall. Cry

j 2 has polygalacturonase activity and is localized in the amyloplast of pollen. Oral administration of the dominant T cell epitope of Cry j 2 inhibited specific T cell responses in Cry j 2-sensitized mice in a mouse model of Japanese cedar pollinosis (Hirahara et al. 1998). Sneezing frequency as a measurable clinical symptom was decreased not only by systemic injections but also by oral administration of the dominant T cell epitope of Cry j 2. To confirm the efficacy of rice-based oral vaccines for inducing immune tolerance against cedar pollen allergens by allergen-SIT, major mouse T cell epitopes derived from Cry j 1 (p 277–290) and Cry j 2 (p245–259) were inserted into the C-terminal highly variable regions of the soybean storage protein glycinin A1bB1b acidic and basic subunits. They were then expressed as fusion proteins in the endosperm of transgenic rice seed under the control of the endosperm-specific glutelin *GluB-1* promoter (Takaiwa 2007). Modified glycinin A1aB1b containing T cell epitopes specifically accumulated to 7 μg/grain in mature dry seeds. In a preclinical model, the proliferative responses of allergen-specific $CD4^+$ T cells, synthesis of specific IgE, and production of histamine were less in mice orally administered with transgenic rice seeds (200 mg) daily for 4 weeks prior to a systemic challenge with crude pollen allergens than in mice fed non-transgenic control rice seeds (Takaiwa 2007). The production of allergy-associated Th2-type cytokines such as IL-4, IL-5, and IL-13 was also inhibited by rice-based T cell epitope peptide feeding. Histamine release from mast cells was also suppressed. Furthermore, allergy symptoms such as sneezing were alleviated after exposure to cedar allergens. These results indicated that mucosal immunization with rice seeds containing T cell epitopes efficiently induced immune tolerance.

Based on this confirmation of the feasibility of an oral peptide immunotherapy program in this animal model, a human version of a rice-based peptide vaccine was developed against cedar pollen allergy. An artificial hybrid peptide 7Crp gene composed of seven linked dominant human T cell epitopes (96 amino acids) derived from the Cry j 1 and Cry j 2 allergens was synthesized using seed-optimized codons for each amino acid (Takagi et al. 2005a). The hybrid 7Crp peptide elicited a positive response in 92% of 48 volunteers with pollinosis without binding to specific IgE, which indicated that it could be used as a safe and effective tolerogen (Hirahara et al. 2001). Notably, the 7Crp peptide had a greater effect on T cell proliferation than that of a mixture of the seven individual T cell epitope peptides.

This 7Crp peptide was specifically expressed in transgenic rice seeds as a secretory protein under the control of several strong endosperm-specific promoters. The GluB-1 signal peptide and KDEL ER retention signal were included to increase the accumulation of the 7Crp peptide. Accumulation of the 7Crp peptide was markedly higher at approximately 60 μg/grain, as determined in CBB-stained SDS-PAGE gels, and accounted for 5–6% of total seed protein. It was mainly deposited in ER-derived PB-I in the endosperm (Takagi et al. 2005b).

Transgenic rice seeds containing 7Crp were orally administered to Cry j 1-sensitized B10.S mice, which recognize only one epitope derived from Cry j 1 as the major epitope. Mice were then nasally challenged with intact Cry j 1 allergen. Both the T cell proliferative response against Cry j 1 and specific serum IgE levels were lower than those in control mice fed non-transgenic rice seeds (Takagi et al. 2005b).

T cell proliferative activity was retained even after boiling 7Crp transgenic rice seeds for 20 min at 100 °C or autoclaving for 20 min, which indicates that oral immune tolerance may still be effective when used with steamed or cooked rice. A safety evaluation of 7Crp transgenic rice seeds showed that the amino acid, lipid, carbohydrate, protein, fatty acid, mineral, and vitamin composition of the transgenic seeds was essentially identical to the non-transgenic control counterpart (Takagi et al. 2006). An oral safety study was performed by administering high and low doses of steamed rice to cynomolgus macaques for 26 weeks (Domon et al. 2009). No adverse effects were observed.

However, since humans with different genetic backgrounds respond differently to various T cell epitopes, peptide immunotherapy using T cell epitopes may not be applicable to all Japanese cedar pollinosis patients in spite of its proven safety. Thus, to treat a broader range of allergy patients, the entire Cry j 1 and Cry j 2 molecules were destroyed by a molecular shuffling and fragmentation process that perturbed the tertiary structure to identify an allergen-specific IgE. The full length of mature Cry j 1 (1–353 aa) was divided into three overlapped fragments. These three fragments with lengths of 131–144 amino acids were inserted into highly variable regions of the acidic subunits of GluA-2, GluB-1, and GluC and were then expressed as fusion proteins with these glutelins under the control of the rice endosperm-specific glutelin *GluB-4* and 16-kDa and 10-kDa prolamin promoters (Wakasa et al. 2013). On the other hand, the coding sequence of Cry j 2 was restructured in the form of a tail to top inverse orientation to disrupt the tertiary structure. This shuffled Cry j 2 was attached to the KDEL ER retention signal at the C-terminus and expressed under the control of the *GluB-1* promoter with its signal peptide sequence (Takagi et al. 2006). A binary vector harboring four expression cassettes (three Cry j 1/glutelin fusions and one shuffled Cry j 2) was introduced into the good-taste rice variety genome lacking three glutelin genes by *Agrobacterium*-mediated transformation (Fig. 12.3). Three chimeric glutelin-Cry j 1 fragments were detected as glutelin precursors with molecular masses from 56 to 60 kDa, but were not processed into mature acidic glutelins containing Cry j 1 subunits. The shuffled Cry j 2 accumulated as a visible CBB-stained band with a molecular mass of 38 kDa. Four individual antigens of 10–25 μg accumulated in one dry grain (approximately 20 mg) and were deposited into ER-derived PB-I. Little or no allergenicity by fragmentation and shuffling was confirmed by binding capacity to specific IgE or the basophil degradation assay (Wakasa et al. 2013).

Transgenic rice seeds (0.6 g) containing accumulations of these destructed whole Cry j 1 and 2 molecules were fed daily to mice for 21 days, which were then challenged twice using a crude cedar pollen allergen. Allergen-specific $CD4^+$ T cell proliferation and IgE and IgG levels were markedly lower than those in mice fed non-transgenic rice seeds (Fig. 12.3). The production of Th2-type cytokines such as IL-4, IL-5, and IL-13 was decreased by the oral administration of transgenic rice grains. Sneezing frequency, which is a clinical symptom of pollinosis, and the infiltration of inflammatory cells in the nasal tissue, such as eosinophils and neutrophils, were also significantly reduced. These results suggest that the oral administration of transgenic rice seeds containing structurally disrupted Cry j 1 and Cry j 2 antigens

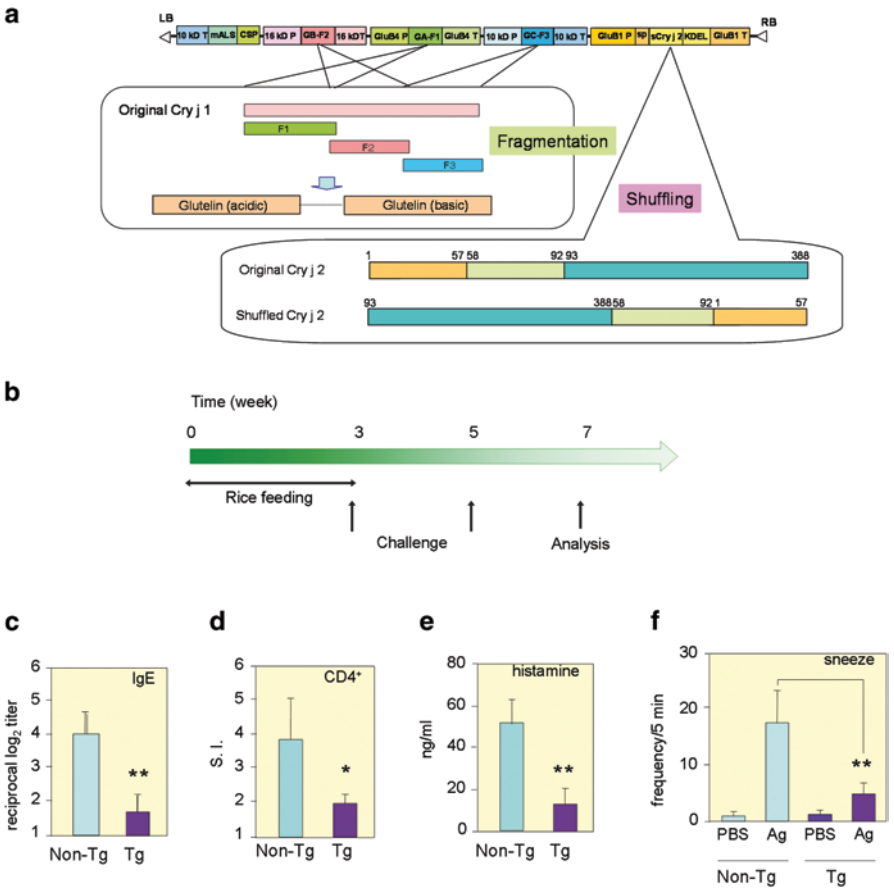

Fig. 12.3 Vector construct used for the expression of hypoallergenic Cry j 1 and Cry j 2 in transgenic rice seeds and the induction of oral immune tolerance by the oral administration of transgenic rice seeds. **a** The Cry j 1 allergen was divided into three overlapped fragments, each of which was inserted into the C-terminal highly variable region of the acidic subunit of three different glutelins. The Cry j 2 allergen was shuffled in the form of a tail to top orientation. The three glutelin/Cry j 1 fragment fusions and one shuffle Cry j 2 were linked to endosperm promoters and were then introduced into the binary vector. Four expression constructs were introduced into the rice genome via *Agrobacterium*-mediated transformation. **b** Experimental time line used for the induction of oral immune tolerance in mice administrated with transgenic rice seeds. **c** Allergen-specific IgE levels. **d** Allergen-specific splenic $CD4^+$ T cell proliferative responses. **e** Serum histamine levels. **f** Frequency of sneezes (sneeze number/5 min after exposure to cedar pollen) sky blue column: mice fed with normal non-transgenic rice seeds, magenta column: mice fed with transgenic seeds

is a more promising approach than with those containing the major T cell epitopes (7Crp) for the induction of immune tolerance against Japanese cedar pollinosis due to its applicability to a broader range of patients.

Conclusions

Plant seeds are a good production platform for tolerogens used in allergen-SIT. Even though destructed antigens or T cell epitopes were produced in seeds, they accumulated securely and at large quantities without degradation. Furthermore, the seed containing the tolerogen acts as an edible vaccine that induces immune tolerance. Tolerogens that accumulate in seeds can be protected from degradation by digestive enzymes in the gastrointestinal tract, resulting in more effective delivery to immune cells in GALT. This is mainly attributed to the bioencapsulation of tolerogens by two barriers, the cell wall and PB, as natural formulation characteristics of the plant cell. Therefore, the oral administration of seeds containing tolerogens is expected to be a promising approach to induce immune tolerance. It is important to note that the immunogenicity of tolerogens stocked in seeds is not lost even by cocking, which is in marked contrast to vaccines against infectious diseases. This is attributed to immunogenicity being fundamentally determined by T cell epitopes between 8 and 15 amino acids in length. Oral administration is simpler and more convenient and comfortable than a conventional subcutaneous injection of the crude extract. Moreover, seed-based edible vaccines are very cost-effective because there is no requirement of downstream processing such as isolation and purification, which represents up to 80% of the overall production cost. The continuous oral administration of tolerogens is more likely to induce immune tolerance than parenteral administration. Furthermore, not only mucosal but also systemic immune reactions are induced by oral administration. Taken together, seed-based allergy edible vaccines represent an innovative allergen-SIT as an alternative to conventional subcutaneous injections.

The efficacy of seed-based allergy vaccines has been demonstrated by their oral administration to model mice. The production of allergen-specific IgE and IgG and T cell proliferation was significantly lower in these mice than in control mice fed non-transgenic seeds. Furthermore, clinical symptoms such as sneezing frequency were downregulated as a result of a decrease in histamine levels. These results indicate that the oral administration of seed-based allergy vaccines can induce mucosal immune tolerance.

However, there are hurdles to overcome prior to the commercialization of seed-based edible allergy vaccines. One concern is entry into the food chain during processing and handling through the process from production to consumption or by gene flow into non-transgenic plants though outcrossing of pollens. Contamination by gene transfer via pollen can be avoided using physical or biological methods. Another concern is the many practical processes regarding the clinical development of pharmaceuticals according to the existing regulations under Good Manufacturing Practice (GMP) regulations (Fischer et al. 2012). Significant changes through discussions with the Japanese Pharmaceuticals and Medical Devise Agency (PMDA) will be required for the commercialization of seed-based edible vaccines.

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Chapter 13
Plant-Based Vaccines as a Global Vaccination Approach: Current Perspectives

Dania O. Govea-Alonso, Edward Rybicki and Sergio Rosales-Mendoza

Introduction

Plant-made vaccines were popularized in the 1990s as an innovative source of "edible vaccines." A number of edible crops such as potato, tomato, lettuce, bananas, corn, and rice were used to express antigens from various pathogens, including *Vibrio Cholerae,* enterotoxigenic *Escherichia coli* (ETEC), *Norwalk virus*, hepatitis B virus, and human and animal rotavirus, among others (Walmsley and Arntzen 2000, 2003; Rybicki 2010). However, although the initial prospect was highly attractive, subsequent research identified problems that would limit the advance in the use of plants as a robust platform for the production of convenient vaccines. Perhaps, the main modification on the focus was the obvious need for introducing some processing in the vaccine formulation to ensure plant biomass stability and proper dosage of the vaccine antigen. Among the technologies that have addressed the identified limitations are the use of bioreactors for biomass production, which allows full containment of the production process; freeze drying of partially purified material; the use of seeds as expression vehicles in order to yield an edible and stable biomass for dosage; and refining of expression systems for higher yields to allow for better purification and formulation of parenteral vaccines (Yusibov and Rabindran et al. 2008). These developments have resulted in sophisticated approaches that are currently making the adoption of plant-based vaccines by the pharma industry a lot more likely than in previous years (Table 13.1). The following section presents some examples of plant-derived biopharmaceuticals adopted by the industry, which illustrate the positive impact of these advances in the adoption of the technology.

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Table 13.1 Limitations and innovations involved in the sophistication of the concept of plant-based vaccines over the last two decades

Aspect	Limitation	Developed alternatives
Low yields	Initial expression strategies resulted in poor protein expression, hampering successful immunization	New expression strategies, such as transplastomic technologies, and transient expression by means of viral vectors, have led to yield improvements in several orders of magnitude, and have allowed for successful immunization of test animals
Poor stability and lack of accurate dosage	Unprocessed plant tissues used in early attempts are labile, which difficulties proper dosage	Stability and dosage are currently controlled by means of using freeze-dried biomass Seed-based expression approaches have also provided higher yields, and dosage can be performed in a straightforward manner by the use of powder derived from ground seeds
Poor immunogenicity/induction of oral tolerance	Oral tolerance is frequently induced when antigens are orally administered, and thus vaccine fails on the induction of immune responses	Adjuvants able to conveniently polarize the immune response have been used Some reports also augur an adjuvant effect attributed to antigen bioencapsulation mediated by plant cells
Establishment of production under good manufacturing practices (GMPs)	Conventional regulations for biopharmaceutical-favored products produced under GMPs Initial focuses of plant-based vaccines did not contemplate this aspect	Procedures under GMPs have been implemented with the modalities of culturing plant cells in bioreactors as well as transient expression systems
Biosafety concerns	Undesired gene flow is associated with the risk of contaminating food chain with antigenic proteins	Transplastomic approaches minimize this risk, since plastome typically shows maternal inheritance Culture of plant cells in bioreactors as well as transient expression system allows for the production under full containment

Successful Cases of Plant-Made Biopharmaceuticals

Influenza Vaccine

One of the most advanced plant-based human vaccines is under development by Medicago Inc. (USA and Canada). Candidate influenza vaccines have been developed by means of expressing the hemagglutinin (HA) protein of H5N1 influenza (A/Indonesia/5/05), as well as of seasonal and H1N1pdm viruses, so as to obtain virus-like particles (VLPs) in a transient expression system in *Nicotiana benthamiana* plants. The candidate H5N1 vaccine is safe and immunogenic when intramuscularly administered to humans, and is currently under evaluation in phase II clinical trial (Landry et al. 2010; Penney et al. 2011). This platform is proposed as an ideal approach for producing vaccines quickly, which is critical for new pandemic influenza strains and viruses such as SARS-CoV and MERS-CoV. It is estimated that plant-based vaccines produced in a transient expression system can be generated within 3 weeks from the release of sequence information.

Anti-HIV Antibody

Although not a vaccine, the EU FP7 Pharma-Planta consortium successfully produced a topically applied anti-HIV monoclonal antibody-based microbicide named P2G12: This was expressed in transgenic tobacco plants, and subjected to a phase I trial carried out at the University of Surrey Clinical Research Centre, UK with the participation of 11 healthy volunteers. Previous studies reported that this antibody is capable of recognizing a cluster of high-mannose-type N-glycans on the HIV envelope protein gp120, leading to a high neutralizing activity both in vitro and in vivo, since it prevents transmission by both parenteral and mucosal routes (Mascola 2002; Veazey et al. 2003). Tobacco plants (*N. tabacum*) producing P2G12 were grown in adequate containment greenhouses at the Fraunhofer Institute for Molecular Biology and Applied Ecology (IME) in Germany (Fraunhofer 2011). At present, no results from the clinical trial are available, but investigators envisage that P2G12 may be used in combination with other plant-produced antibodies for the formulation of a broadly protective vaginal microbicide (Pharma-Planta 2011).

Therapeutic Enzyme for Gaucher's Disease

This example constitutes the first plant-derived biopharmaceutical approved for use in humans, illustrating their potential to reach the marketplace. Protalix Biotherapeutics (Carmel, Israel) established the production of a human recombinant glucocerebrosidase in suspension cultures of transgenic carrot cells (*Daucus carota* L.). This is a therapeutic enzyme for Gaucher's disease, a lysosomal storage disorder

caused by mutations in the human glucocerebrosidase gene. Unlike the Chinese hamster ovary (CHO) cell-derived product, the carrot-derived enzyme has terminal mannose residues on its glycans, allowing for efficient uptake via macrophage mannose receptors. Phase III clinical trial was completed in 2009, and orphan drug designation has been granted from the Food and Drug Administration (FDA). The product is currently approved for human use by health agencies from a number of countries (Protalix 2011).

What Is Next in the Field of Plant-Based Vaccines?

The cases mentioned above exemplify how plant-based platforms have shown a sufficient degree of maturity in development as well as robustness, leading to modalities of production that are finding their niche in the biopharmaceuticals industry.

It is clear that production of biopharmaceuticals such as antibodies or enzymes are prone to less complex evaluations than the case of vaccines, where complex parameters such as achieving long-lasting immune responses capable of supporting immunoprotection should be addressed. Therefore, it is envisioned that vaccines, whose target antigens, immunogenic properties, and conventional large-scale production and production processes are well established, will be the first plant-based vaccines to be produced in transient systems, as this implies a standardized and approved path for the development of biosimilars. An influenza virus vaccine is the case that highlights this potential. However, the reluctance of Big Pharma to move from conventional production platforms to those of the new generation will still be a hurdle to overcome.

The road to reaching the market with plant-based vaccines and deriving a substantial exploitation of this technology is still a long one. The following section summarizes perspectives that are identified at both the basic-research and the regulatory industrial levels for pursuing this goal through technical innovations that meet the regulatory requirements, promoting social acceptance in parallel, and the politics of exploiting the technology in poor countries.

Perspectives in the Context of Basic Research

Advancing on the Development of Oral Formulations

A major challenge in the field of plant-based vaccine development has been in the modality of oral immunization with plant biomass. The high potential is attributed to this immunization approach, such as simple needle-free delivery, very low costs because minimally processed plant biomass is administered as the vaccine, and the possibility of inducing mucosal responses which can protect against orally or sexually transmitted diseases. However, several factors need to be addressed before the successful elicitation of safe and robust immune responses through oral immunization is assured.

One challenge consists of eliciting robust immune responses by the oral route, which is hampered by both antigen degradation and tolerogenic nature of the system (Fujkuyama et al. 2012; Mestecky et al. 2008). Several factors may impact the immunogenicity of oral vaccines. Among these, formulation is a critical aspect (Gebril et al. 2012). In the case of plant-based vaccines, it is proposed that a protective effect is exerted by the plant cell wall, which is termed as a "bioencapsulation" effect. Interestingly, a number of studies have revealed higher immunogenicity when the antigen is administered in the form of a plant-derived preparation rather than as a purified soluble antigen (Rosales-Mendoza et al. 2011; Hayden et al. 2012; Pniewski et al. 2011), suggesting a matrix effect that may be associated with (1) a delayed degradation rate or (2) the adjuvant effects exerted by plant compounds (Otsuki et al. 2010).

However, the reality is that no systematic studies to characterize this effect have been conducted, with only a few reports that have focused on studying the bioavailability of oral plant-based vaccines and the effect of the plant tissue used for expression and immunization (e.g., Pelosi et al. 2011, 2012). Thus, future evaluations of the effect of vaccine formulation will provide new insights on the bioencapsulation effect and optimization of the immunogenic potential of this kind of vaccine. Among the parameters to be studied are particle size, pill/capsule composition, and the type of plant tissue to be administered.

It is well known that plants synthesize a complex population of metabolites that sometimes exert profound biological effects. Some of the plant compounds that are recognized as immunomodulators are the following: flavonoids, terpenoids, and saponins (Potterat and Hamburger 2008; Castro-Díaz et al. 2012; Schepetkin and Quinn 2006). Some plant compounds can also act as mucoadhesive agents, accounting for more efficient antigen uptake (Garg et al. 2010). Thus, a relevant research objective in this field would be the characterization of specific plant compounds responsible for enhancing the immunogenicity of the plant-derived formulations: this is of particular relevance, as a significant challenge in the field of oral vaccination consists of providing appropriate adjuvants, which is in general accomplished by high-cost formulations.

Another concern around the immunological events associated with oral vaccines is the possibility of breaking tolerance towards food proteins present in the plant delivery vector. One of the few studies focused on this issue was published by Nojima et al. (2011). They observed that humoral responses against rice proteins were elicited when rice expressing a chimeric protein comprising cholera toxin B (CTB) and an Alzheimer's disease-related antigen were orally administered to mice. However, the authors hypothesized that immunological tolerance against those non-target rice proteins could be induced by means of breast-feeding. Therefore, immunized mice were subsequently fed by lactating mothers who had consumed rice proteins. This led to a successful induction of tolerance, suggesting that the design of specific immunization schemes has a potential for modulating undesired immune responses. However, this field of investigation is still in its infancy, which highlights the need for expanding not only the evaluation target immune responses and challenge experiments in animal models but also addressing questions regarding safety in terms of the elicitation of responses against non-target proteins. Systematic research in

this direction must be conducted to better understand how immune responses elicited by plant-based vaccines could be modulated in order to avoid undesired immune responses that may eventually mediate allergic events and other undesirable side effects.

Expanding the Modalities of Expression of Immunogens

Although current expression strategies are considered robust in terms of yields suitable for industrial applications, certain aspects of the expression systems may allow for innovative and improved formulations. The induction of broader immune responses is a relevant goal, especially in the case of hypervariable pathogens (e.g., influenza or HIV viruses). One approach to address this is the simultaneous expression of several antigens from a single transformation or transient event. When a transplastomic technology is used, the use of polycistronic vectors can allow simultaneous expression of proteins (see Modalities for Expression of Antigens in Plants: Plastid-Based Expression Strategies). In addition, some virus-derived vectors may allow this in nuclear expression approaches. One such strategy involves the use of internal ribosome entry site sequences (IRES), which allow for the translation of several open reading frames (ORFs) via CAP-independent translation (Ha et al. 2010; Gouiaa et al. 2012). Another alternative is the use of the picornaviral 2A endopeptidase sequence, which mediates a translational skip mechanism, allowing the production of different polypeptides from a single ORF where target antigens are linked by a 2A sequence (Halpin et al. 1999; Ha et al. 2010). These approaches remain essentially unexplored in the field and are considered to have great potential to aid in the improvement of plant-based vaccine production.

Diversifying Full-Contained Production Platforms

Among the options for implementing processes under full containment and good manufacturing practices (GMP) are the use of cell suspensions and organ cultures such as hairy roots systems. These approaches offer tightly controlled bioprocesses where environment concerns related to undesired gene flow are eliminated (Franconi et al. 2010; Michoux et al. 2011; Skarjinskaia et al. 2013). In addition, some platforms implemented in the biopharmaceutical field remain to be explored for vaccine production. In particular, moss (*Physcomitrella patens*) has been proposed by Rosales-Mendoza et al. (2013) as a robust platform for the production of recombinant vaccines. This non-vascular plant can be propagated in a filamentous development stage, named protonema, which can be grown in liquid media using bioreactors. This approach has several singular advantages, such as a low cost and well-established production system a platform for the production of plant-based vaccinesem, full containment of production, efficient secretion of the antigen to the media (facilitating purification), and the possibility of producing specific glycoforms by the use of strains genetically engineered for alternative glycosylation

machinery. Exploring emerging plant production platforms may lead to innovative developments, for example, the production of immunogens with specific glycosylation patterns with improved immunogenic properties, and new processes performed under full containment.

Targeting a Broader Number of Diseases

A number of highly relevant pathologies considered as vaccine preventable still cause significant epidemiologic impact. As costs are in general the main obstacle to widespread vaccination, the adoption of the plant-based technologies for the development and production of vaccines for an expanded list of vaccine preventable diseases represents an important field of opportunity for medical biotechnology. The following section describes some diseases for which new vaccines are urgently needed, and which constitute logical targets for the plant-based vaccine production technologies.

Tropical neglected diseases. This group of diseases affects the lives of 1 billion people worldwide, but no vaccines are available for them (WHO 2010). Vaccines against neglected tropical diseases (NTDs) should be of low cost and preferably needle free, in order to reduce the logistic cost of their administration. Although a number of efforts on developing vaccines to fight rabies, cysticercosis, dengue fever, and helminthiasis have been reported, there is still a need for developing plant-based vaccination models for more of this group. Development of plant-based vaccines against tropical neglected diseases is identified as a key priority for exploiting this technology, as low-cost formulations may be produced to fight these diseases that mainly impact low-income populations ((Rosales-Mendoza et al. 2012a).

Non-communicable diseases. These pathologies killed tens of millions of people in 2008, and a high fraction of these deaths occurred in people under the age of 60 years, comprising the most productive human cohort. The incidence of these diseases continues to rise, especially in low- and middle-income countries. Of particular interest in combating these diseases are vaccines against cancer, hypertension, diabetes, and atherosclerosis, which play a major role in the mortality rates at the global level (WHO 2011). According to the World Health Organization (WHO), the leading global risks for mortality are high blood pressure (responsible for 13 % of deaths globally), tobacco use (9 %), high blood glucose (6 %), physical inactivity (6 %), and overweight and obesity (5 %). These risks are responsible for raising the risk of chronic diseases such as heart disease, diabetes, and cancers (WHO 2009).

Type I diabetes is an autoimmune disease that has been targeted by a number of groups using plant-based vaccine models. The proposed therapy consists of eliciting tolerance against the glutamic acid decarboxylase, which is a self-antigen associated with the development of immune responses responsible for diabetes development. This goal is pursued by administering autoantigens by the mucosal route, which is "tolerogenic" by nature. This kind of approach has been successfully evaluated in animal models with promising findings in terms of therapeutic effects, suggesting a considerable potential for clinical trials (Alvarez et al. 2013; Langridge et al. 2010).

Hypertension and atherosclerosis. Immunotherapies for the treatment of these pathologies are well documented. In particular, vaccination models using conventional formulations have been based on the induction of humoral responses against physiological proteins whose elevated levels favor the development of the pathology (Bachmann and Jennings 2011). However, plant-based vaccines against hypertension and atherosclerosis have been recently suggested (Rosales-Mendoza 2012b; Salazar-González and Rosales-Mendoza 2013).

Cancer. Interestingly, plants have served as a source of recombinant antibodies for use in the treatment of non-Hodgkin lymphoma. Recombinant idiotype-specific personalized vaccines have been developed by the expression of tumor-derived single-chain Fv (scFv) antibodies in *N. benthamiana* plants by means of a TMV-based expression vector (McCormick et al. 1999). Autologous full-idiotype IgG-based vaccines have been produced by *N. benthamiana* plants using the transient TMV-based magnICON platform (Marillonnet et al. 2004). Mice subcutaneously immunized with this vaccine were protected against lethal tumor challenge. Patients with follicular lymphoma have been enrolled for a phase 1 clinical trial of the full-idiotype vaccine (sponsored by Bayer Innovation GmbH.http://www.clinicaltrials.gov; NCT01022255 ; NCT01022255).

However, active immunization therapies have essentially not been developed for most other human cancers. This represents a field of opportunity, as conventional chemotherapy and radiotherapy lack specificity and show significant toxicity. Vaccines are proposed as complementary treatment. For example, the human carcinoembryonic antigen (CEA), which is over-expressed by a large number of epithelial neoplasias, including colorectal carcinoma (CRC), gastric, pancreatic, breast, lung, and ovarian carcinomas (Hammarstrom 1999), is considered a tumor-associated antigen that can be targeted in therapeutic cancer vaccines (Berinstein 2002). This has been shown to be safe, and induction of antigen-specific immune responses has been achieved (Mosolits et al. 2005; Samanci et al. 1998). It is thus expected that focusing on this kind of target will be of significant relevance in the field of plant-based vaccines.

The Regulatory Framework

In spite of the many candidate vaccine antigens expressed in plants, and those proven to be effective thus far, most candidates have not progressed beyond the preclinical phase. Factors that limit their progress to the market will be further elaborated on in this chapter.

Interestingly, a regulatory framework has already been established to limit the growth of crop plants expressing pharmaceuticals, including vaccines. This development limits the use of, for example, maize, rice, potatoes, tomatoes, and other foods for commercial vaccine production. Commercialization also requires facilities for manufacturing vaccines on medium and large scale under current GMPs (Streatfield 2005). These GMPs were established by the WHO, and constitute an

associated group of norms and activities to guarantee that every product meets and retains the characteristics of design required for its use. The GMPs minimize unanticipated risks that may occur during the screening of the final product (van der Laan et al. 2006). For example, Kentucky BioProcessing, LLC (KBP; Owensboro, KY), is a facility specialized for the expression, extraction, and purification of recombinant proteins from plants, from bench to commercial scale, using its proprietary Geneware® expression technology under the GMP conditions (Yusibov et al. 2011).

The evaluation of vaccines by traditional methods can be followed for plant-based vaccines. However, plant containment is a particular issue for the production of this type of vaccines. In terms of production and clinical assessment, plant-based vaccines must apply for the Investigational New Drug (IND) application and all applicable regulatory requirements (Yusibov et al. 2011). These guidelines are contained in draft documents defined by the FDA and US Department of Agriculture (USDA), which address quality aspects in the production of plant-based biopharmaceuticals for human and animal use, and present the points related to product safety and efficacy, environmental issues, and manufacturing control (Center of Veterinary Medicine et al. 2002).

In particular, FDA establishes that development stages must comprise evaluation of: (1) the presence of potential allergenic or toxic compounds, (2) the method of plant production and propagation, (3) the characterization of recombinant DNA, and (4) genetic stability for those cases based on stable transformation events. Environmental concerns should be taken into consideration by means of implementing confinement mechanisms in order to not only control the spread of the bioengineered pharmaceutical plants but also meet regulations for transnational commercialization.

In addition, facilities and procedures should be designed to prevent cross-contamination of the source material during harvest and processing, which implies establishing procedures for appropriate cleaning, maintenance, and sanitization of equipment and utensils. Thus, malfunctions or contaminations that would alter the safety, identity, strength, quality, or purity of the products are prevented. On the other hand, testing the presence and identity of potentially harmful constituents is a requirement for performing preclinical trials. These comprise toxins, pathogens, pesticides, herbicides, fungicides, heavy metals, anti-nutrients, and allergens, which are assessed by in vitro and in vivo assays. In addition, unintended immunogenicity due to plant-specific posttranslational modifications must be assessed.

Once passed to clinical trials, existing guidelines for the clinical evaluation of drugs and biologics for humans are considered, and additionally, specialized advice can be obtained by communication with regulatory agencies, such as the Center for Drug Evaluation and Research of the FDA (CDER) or Center for Biologics Evaluation and Research of the FDA (CBER; Center of Veterinary Medicine 2002).

Toxicity, dose, lot-to-lot consistency, possible allergic responses, and immune tolerance must be evaluated during the development of plant-based biopharmaceuticals intended for commercialization. However, in spite of the inherent potential of plants as bioreactors, robust proofs would be provided regarding efficacy, ease of delivery, and cost. Because of these stringent regulatory requirements for human

products, veterinary products are probably going to be the fastest to be marketed (Hammond and Nemchinov 2009).

Despite the fact that oral vaccination is considered to be an advantageous immunization approach, this approach faces a number of challenges. A suitable dosing regimen must be defined for each vaccine candidate; the formulation of oral vaccines requires, in general, high doses of antigen, which in turn may also be difficult to be accurately determined because of the complexity of the gastrointestinal tract (see Mucosal Immunology and Oral Vaccination). Therefore, formulation and delivery strategies should be optimized to attain immunoprotection under acceptable consistency (Streatfield and Howard 2003).

It is also a consideration that ethical issues for plant-based vaccines have not been subjected to sufficient analysis. Many of the ethical and social debates related to genetically modified (GM) plants have covered the topic of plant-based vaccines in a superficial manner, if at all. However, the negative perception of GM foods may in fact influence the social acceptability of plant-based vaccines. The main reason that GM foods have not been widely accepted by civil society is the perception that their consumption carries unknown risks, and that the major beneficiaries are farmers and seed companies, not the final consumer.

In contrast to these examples, under humanitarian projects such as those of the Pharma-Planta consortium, the major beneficiary of plant-based vaccines and therapeutics will be patients in the developing and poor countries. However, substantial transfer of the technology, its principles, and its benefits will definitively favor a positive perception of the technology, and hopefully its eventual acceptance.

Commercialization of Plant-Based Vaccines

Interest by and investment from the pharmaceutical industry are seen as key factors for the benefits of the plant-based technology for biopharmaceutical production to become a reality. During the last two decades, the production of biopharmaceuticals has increased notably. In the period from 1982 to 1991, 15 biologics were approved by the FDA, while 54 biopharmaceuticals were approved from 1992 to 2001. During the last two decades, a total of 95 biopharmaceutical products have been approved by regulatory agencies for treating several human diseases (Goldstein and Thomas 2004; Rader 2009; Wong 2009).

Recombinant proteins produced in plants have found a preferential niche in the biopharmaceutical field, rather than in industrial products such as enzymes and polymers. However, pharmaceutical companies have not completely adopted biopharmaceuticals as the next-generation drugs (Davies 2010). Molecular farming approaches have been technically successful, and possess the potential for being implemented on a large scale, as evidenced through many publications, patents, and field tests. These studies have yielded positive results in efficacy trials in animals, and some of them have been evaluated in clinical trials with promising insights (Tiwari et al. 2009; Yusibov et al. 2011).

Plant-based vaccines have a high potential for improving global health, although this will depend on demonstrations of efficacy, safety, and feasible commercialization, for pharmaceutical companies, philanthropic organizations, or the governments of developed or developing countries. Sadly, and despite their promise, breakthroughs in the development of oral vaccines have been few and far between, due partly to the complexity of the approach. Sustained collaborations between plant scientists, immunologist, and vaccinologists are considered of particular importance in maintaining the advancement in the development of effective and cost-effective oral vaccine candidates. This requires not only innovative research goals to address important questions related to safety and strategies to improve immunogenicity but also significant economic resources through public and non-profit organizations, as well as investments from industry in order to invigorate this field (Tiwari et al. 2009).

Notably, a vaccine for *Newcastle virus* produced in a suspension-cultured tobacco cell line by Dow AgroSciences, India, was successfully tested as a purified injectable product in chickens, and was approved by the USDA with full licensure for animals in the USA (Rybicki 2009; Melnik and Stoger 2013). The most advanced approach for plant-based human vaccines is in development by Medicago Inc. (USA and Canada), consisting of influenza candidate vaccines targeting seasonal H5N1 and seasonal and H1N1pdm influenza (Landry et al. 2010; Penney et al. 2011; Medicago Inc 2013).

The support of public funds is needed in order to advance the development of plant-based vaccines to the stage at which corporate investors will become involved. However, it is also necessary that they be evaluated by clinical trials if this technology is to prove itself worthy of the major disbursement of funds granted to target particular diseases in specific locations (Robert and Kirk 2006).

The Outlook for Developing Countries It is envisioned that plant-based biopharmaceutical industry will be consolidated in the next decade. Recombinant protein vaccines, including subunit-based or VLP-based vaccines, are good candidates for these new production systems, and plant-based pharmaceutical companies have responded to regulatory requirements in order to demonstrate that plants are an appropriate platform for production (Davies 2010; Scotti and Rybicki 2013).

In a realistic view, translational companies will favor financial support for developing vaccines with a significant market. Thus, in spite of achieving significantly at the preclinical level, it is considered of particular relevance to advance this technology in developing countries where the need for new vaccines against diseases is great, and is neglected by the larger companies.

A humanitarian focus is an indispensable factor in opening the path for exploitation of plant-based vaccines. As an example, the Pharma-Planta consortium was established in Europe in order to promote the production of anti-HIV microbicidal antibody candidates meeting all regulatory requirements, GMP standards, and preclinical toxicity testing, with a statement of intent for humanitarian use, which guaranteed access by poor countries to the plant-based products to be developed (www.pharma-planta.net/). Of particular relevance are the objectives of this consortium that comprised (1) the development of robust risk-assessment practices

for plant-made pharmaceuticals, based on health and environmental impact, collaborating with regulatory authorities within the EU and public groups to guarantee safety and acceptance of the production systems based on biosafety regulations and (2) design and implementation of a program intended for securing and managing intellectual property, and thus facilitation of the availability of high-priority plant-derived recombinant pharmaceuticals to poor countries, while at the same time allowing the products to be developed commercially in Europe and North America. This example illustrates the kind of efforts that may allow for a massive exploitation of the technology, and which will aid in overriding the "Valley of Death" that blocks the wider use of technologies for humankind benefit (Obembe et al. 2011).

It is envisioned that poor or developing countries must establish policies favoring increased connections between research institutes and universities with local companies or even government agencies: This will be a critical factor for exploiting the potential of plant-based vaccines in countries where the need for low-cost vaccines is great and urgent. Novel methods are needed to finance the increasing number of new vaccines that have the potential to save lives in countries that are too poor to afford them (Hefferon 2013; Levine et al. 2011). Selection of new vaccines that should be a priority for particular countries should be given under the advice of the WHO, in conjunction with a national immunization advisory committee, as adoption of plant-based vaccines could necessitate the modification of immunization schedules and delivery procedures.

It has recently been suggested (Rybicki et al. 2013) that developed country-firms and non-governmental organizations concerned with vaccine manufacture should team up with developing country-scientists and institutions for both research and development, as well as for clinical trials. There are already indications that this is happening: for example, Fiocruz/Bio-Manguinhos (Brazil) is collaborating with iBio Inc., (USA) to make yellow fever virus vaccine using plants and Ventria Bioscience (USA) has been growing transgenic rice-producing lactoferrin and lysozyme for some years now (see Rybicki et al. 2013). This kind of collaboration could open the door for the production of low-cost vaccines and other pharmaceuticals where they are needed, using local scientific and commercial resources, which would be a welcome change from the present model.

Concluding Remarks

Plant-based production has evolved into a robust approach for manufacturing vaccines under conditions that meet the requirement of a variety of regulatory systems. The benefits derived from this technology are expected to become evident in the coming years and to be realized by the introduction of the first vaccines into the market. Non-vaccine biopharmaceuticals already in the market are an indication of the potential for achieving this goal in the short term. Interesting aspects of the development of oral vaccines represent a research path that should be explored in a detailed and systematic manner in order to accelerate their development; these

constitute the ideal application of plant-based vaccines due to easy administration and low costs.

Humanitarian initiatives such as Pharma-Planta consortium are identified as key strategies for attaining the benefits of biofarming for developing countries. Future research efforts to expand the application of this technology to new target-relevant diseases will also be of key importance to exploit in a wider manner the advantages of plant-based vaccines.

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