Sergio Rosales-Mendoza *Editor*

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

An Integrated View

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Springer

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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.

Sergio Rosales-Mendoza
Editor

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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

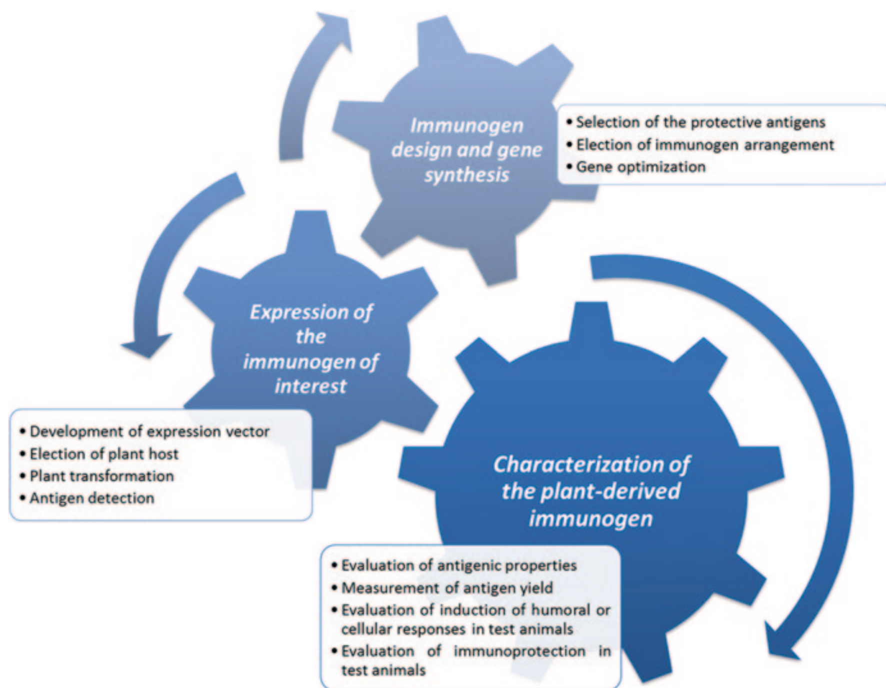

Fig. 1.1 Steps in the development of plant-based vaccines

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).

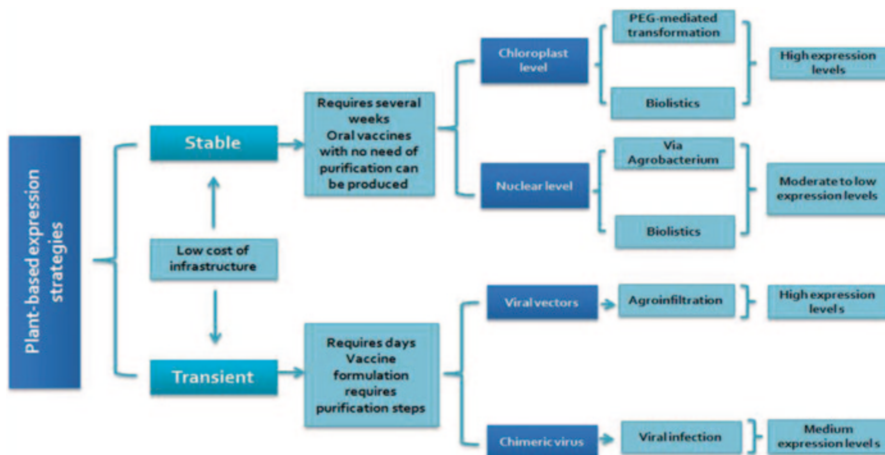

Fig. 1.2 Summary of the strategies for the plant-based expression

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

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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

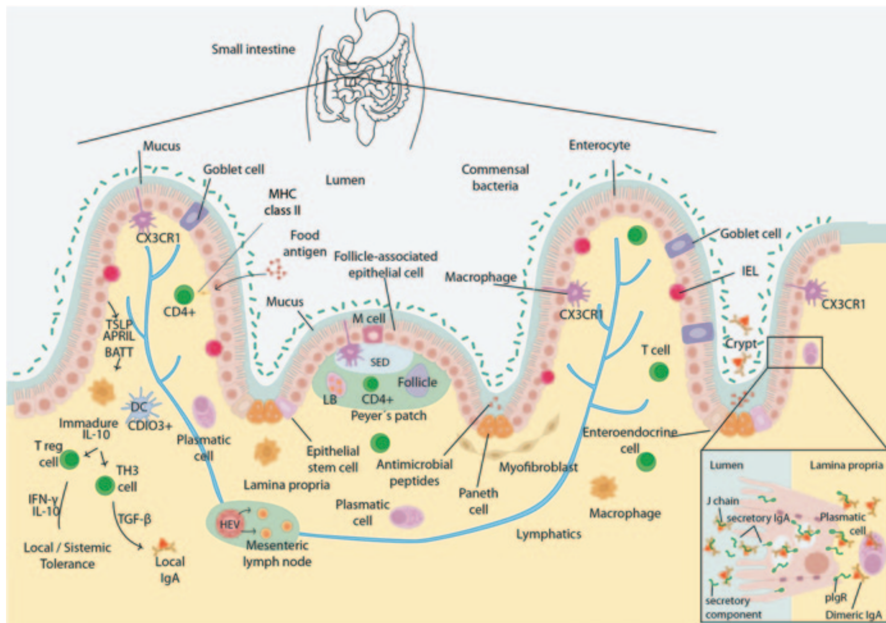

Fig. 2.1 Anatomy and homeostasis of the intestinal immune system. The gut-associated lymphoid tissue (GALT) can be divided into inductive and effector sites, which consist of organized and diffuse lymphoid tissues, respectively. The organized tissues are the Peyer's patches (PP) and mesenteric lymph 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).