Biotechnology in Agriculture and Forestry 68

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Series Editors

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Commercial Plant-Produced Recombinant Protein Products

Case Studies

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Editors

Commercial Plant-Produced Recombinant Protein Products

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Dedication
In Memory of Michael Horn (1955–2012)

In 2012 the scientific community lost one of its most dedicated scientists in the field of plant cell culture and genetics, Dr. Michael Horn. Dr. Horn succumbed after a short illness to amyotrophic lateral sclerosis (ALS), sometimes referred to as "Lou Gehrig's disease." Dr. Horn spent his career discovering the scientific principles that have been applied to a variety of purposes including plant propagation, somaclonal variation, and recombinant expression of foreign proteins in plants.

Dr. Horn received his B.A. in 1977 and MA in 1980 from the Department of Biology, University of Missouri. He then moved to the Department of Agronomy, University of Illinois, where he received his PhD in 1984 working with Dr. Jack M. Widholm on the "Establishment, Optimization and Characterization of Photoautotrophic Soybean Suspension Cultures." He continued to pursue his research passion throughout his career, albeit in many different companies due to the flux in the biotechnology industry. These included Ciba-Geigy, Plant Genetics/CalGene, Agrigenetics/Mycogen, ProdiGene, Applied Biotechnology Institute, Targeted Growth, and Cibus.

Dr. Horn used his technical skill to develop improved methodology for cell cultures, transformation, and gene expression. He then applied these methods to a number of different crops (e.g., rice, peanuts, corn, tobacco, and orchard grass) and to a number of different projects (improved agronomic traits, improved food quality, and production of pharmaceutical and industrial proteins). While the scientific community, along with his family, will miss his personal and professional contributions, he will not be forgotten. Many of the chapters in this book describe work that is either a direct or indirect result of Dr. Horn's contributions.

Throughout his career in industry, he kept engaged in scholarly activities including being an author or inventor on over 35 publications and patents. He was involved in several professional organizations, most

notably in the Society for In Vitro Biology (SIVB; formerly TCA) where he was editor of ExPlants and secretary and president of the plant section. He was also Publications Committee Chair and as such brought Springer in as publisher of In Vitro Plant and In Vitro Animal.

Dr. Horn will be remembered by many for his scientific achievements but those that had the opportunity to know him personally will undoubtedly have special recollections of his presence. He continually brought up new ideas to pursue but was extremely tolerant to others when they did not agree with his analysis. He worked steadily throughout any project always trying to make it succeed despite the many roadblocks that are frequently encountered in new fields of research. In short, he behaved as the rest of us strive to act, as model scientists.

In addition to his professional activities he never abandoned his responsibility as a husband, parent, and grandparent. These personal connections always kept him well grounded to the most important aspects in his life. While these gave him a considerable amount of enjoyment, they also provided him with support for his professional obligations. This was most apparent from the tireless work of his wife, Patricia. She provided support for him over his entire career but her dedication was most obvious after he was diagnosed with ALS. While his mind was as sharp as ever, he could not physically do many simple tasks. Patricia therefore provided him with the physical support he needed including driving

him to work, reading, translating, and writing letters when he could no longer physically do these himself.

During this entire time we knew him, he was never without hope including in the later stages of his disease. Even toward the end of his life, after he had lost all mobility and much of his speech, he accepted his limitations but continued to do whatever he could for the family and the scientific community while never losing his sense of humor. He has left a legacy for many of us to emulate as an outstanding role model to balance both a professional career and a personal life. We dedicate this book to his memory.

Preface

Large-scale protein production has come a long way with the onset of recombinant DNA technology in the 1980s. Initially microbes, such as bacteria and yeast, were the choice of host used to produce commercially important proteins; their short generation time and growth to high densities in bio-fermenters were valuable traits. As technology became more sophisticated, other hosts such as cell lines, animals, and plants were explored. Plants lagged behind most other systems primarily because initial biotechnical work focused on agricultural improvement to crops rather than their use for the expression of novel products.

Attention has since turned to using plants as hosts to produce commercially important proteins. Many reviews have been written about the theoretical aspects of this topic but the present volume is focused on commercial successes: case studies of projects that have commercial potential or products that have already been commercialized, illustrating the advantages that plants can have over bacterial, fungal, or animal cell culture hosts. These case studies demonstrate the hurdles that must be overcome and the benefits of using plants to produce industrial and pharmaceutical proteins as well as vaccine antigens. It is predicted that plant protein production is the beginning of a new paradigm for the commercial production of proteins that over the next decade will expand dramatically.

The commercialization of plant-produced proteins has progressed slowly over the past 15 years since the first introduction of a commercial product demonstrating feasibility. Many factors have contributed to this slow progress, but, in brief, the technology was not robust and predictable in the early stages to compete on a strictly cost basis with other existing platforms, and there was little motivation to fund technology improvements to a system that was considered a threat to existing platforms. In the last several years, however, the advantages of plant production systems beyond the unit costs are enabling the acceptance of the technology. The clear front-runner is the move into an animal-free source of proteins for cell cultures. This may soon be followed by an animal-free source of therapeutics, a rapid system for the production of parenteral vaccines, orally delivered vaccines, and industrial enzymes that can only be produced on the scale that a plant system can provide. The advantages of plant-produced proteins beyond the unit cost are the

key to the initial commercialization. In the longer term as the technology becomes more engrained into the industry, this approach can be used for a variety of other proteins where plants can compete on unit cost as well.

In this volume, the focus is on products from plants that have either been commercialized or that are near commercialization. We have chosen protein products that illustrate the promise of the system, for example, highly purified proteins free of concerns over animal pathogen contaminants, directly delivered proteins such as orally delivered vaccines, or minimally processed industrial products.

This book is divided into four parts. The first part on *highly purified proteins* describes trailblazing technologies that are effective for the production of proteins at commercial production levels, at pharmacological and research-grade purities. Some of these proteins are toxic to cells when expressed at even moderately high levels, so they represent a major advance in strategies for the production of proteins that may interfere with normal cellular pathways. These strategies may be modified for use in non-plant systems.

The second part on *vaccines* examines strategies for administration of plant-produced antigens through oral and parenteral routes and for human and veterinary applications. The failure of straightforward approaches to vaccine production for pathogens that show antigenic drift has been addressed by the use of novel strategies such as transmission blocking vaccines, and these strategies may be extrapolated to other vector-transmitted diseases. Antigens that are presented in a structural form that resembles the pathogen are also examined. For veterinary application, vaccines effective for use in domestic herds and wild animals are examined. Some of the outcomes pursued are effectiveness, rapid production, cost-effectiveness, and ease of administration.

The third part on *industrial proteins* evaluates the production of proteins that have applications in the paper and food industries. A unique feature of these proteins is that they can perform their purpose without purification to homogeneity. Cellulase enzymes are effective for conversion of cellulose to biofuels but also for making wood amenable for conversion to paper pulp without the use of environmentally unsafe chemicals. Thus, the indirect effects of the use of these enzymes are also beneficial.

The final part on *future directions* examines the benefit of plants as hosts and reviews some of the possible applications and the regulatory and public perspectives with regard to their use.

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Chapter 1
Introduction: Plant-Produced Protein Products

Elizabeth E. Hood and Paul Christou

1.1 A Short History of Recombinant Protein Production in Plants

Recombinant protein production in plants encompasses vaccines, pharmaceuticals, and industrial proteins. Within each of these categories are numerous products and host systems with applications to multiple diseases and industrial processes. This industry requires gene transfer from other organisms into plants and allows the plants to overproduce the proteins for the desired application.

Several companies and university laboratories have had programs in plant expression of proteins over the past two decades. The plant biotechnology companies that are focused on production of those proteins are listed in Table 1.1. Significant effort has gone into developing these new products using several plant systems. The choice of system depends on many factors including the type of protein, the technology utilized, the platform of the company, and the funding source (Howard and Hood 2005). Several of these companies are still functional, and others have closed but reemerged as new entities.

1.2 Advantages of Using Plants

Compared to animal and microbial systems, the advantages of using plants for protein production are numerous. For example, plants do not harbor animal pathogens, which is particularly advantageous for pharmaceuticals and vaccines

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Table 1.1 Protein production companies, crops, and product foci over the last three decades

Company	Crop	Main products	Status	Comments
ProdiGene	Maize	Pharma, vaccines, enzymes	Development	Inactive
EpiCyte	Maize/tobacco	Antibodies	Development	Out of business
SemBioSys	Safflower	Pharma, vaccines	Clinical trials	Out of business
Meristem	Maize/tobacco	Lipase, lactoferrin	Development	Out of business
Crop Tech	Tobacco	Enzymes for ERT	Licensed to Protalix and Pfizer	Out of business
Biolex	Lemna	Pharmaceuticals	Research only	Out of business-some products in development by Synthon
Medicago	Transient tobacco	Pharma, vaccines	In production	Company sold to Mitsubishi
Planet Biotechnology	Stable tobacco	Anthrax antitoxin	Successfully protects animals in trials	Planning clinical trials
Ventria	Rice	Blood proteins and therapeutics	Selling research products	Human clinical trials in process
Icon Genetics	Transient tobacco	Biotherapeutics and monoclonal antibodies	Clinical trials in process	Acquired by Nomad
Syngenta	Maize	Amylase—enolase for corn ethanol	In production	First industrial output trait deregulated
Applied Biotechnology Institute	Maize	Cellulases, HepB vaccine, brazzein	Development	Licensed ProdiGene technology
Infinite Enzymes	Maize	Cellulases	Reagent sales	Other enzymes in pipeline
BioStrategies	Transient tobacco	Pharma enzymes for ERT	Development	SBIR funded
Caliber Biotherapeutics	Transient tobacco	Vaccines and monoclonal antibodies	Preclinical trials	Production with G-Con pods
Fraunhofer MBC USA	Transient tobacco	H1N1 and malaria vaccines	Preclinical and clinical trials	Nonprofit organization
Kentucky BioProcessing (formerly LSBC)	Transient tobacco	Aprotinin, vaccines, pharmaceutical	Used in research and cell culture	In production
Mapp Biopharmaceutical	Transient tobacco	Monoclonal antibodies	Preclinical trials	Made by Kentucky BioProcessing

Protalix	Carrot cell culture	Glucocerebrosidase	In production for Gaucher's syndrome	Primarily Israeli market
ORF Genetics, Iceland	Barley	Human growth hormone, cytokines	Diagnostics, research, cosmetics	In production from seed
Dow Agrosciences	Cell culture	Newcastle disease viral vaccine	Approved	Unknown if company is selling
Inserogen	Tobacco	Vaccines and biotherapeutics	Development	Start-up

ERT enzyme replacement therapy, *LSBC* Large Scale Biology Corporation

(Ramessar et al. 2008; Sabalza et al. 2011). Pathogen-free pharmaceuticals are desirable whether delivered orally or through injections. Thus, the plant host can be a food crop, such as corn, canola, or rice, or a nonfood crop, such as tobacco.

Because several of the plant hosts are food plants, oral delivery of the proteins for therapeutic purposes is possible. Oral delivery has been demonstrated for potato (Tacket et al. 1998), corn (Lamphear et al. 2004; Hayden et al. 2012), and banana (Mason et al. 2002). In each case, the integrity of the protein must be ensured through the formulation process, e.g., extrusion or cooking. If raw, the plant host must be edible without processing, such as a fruit or vegetable. In contrast to injected pharmaceuticals, the cold chain may not be required to transport these orally delivered products to the target population, which is particularly useful when serving developing countries. This is a distinct advantage for plant systems—high product stability at ambient temperatures.

Direct addition of the proteins in their host tissue may be possible without the need for purification. This can be an advantage for pharmaceuticals as well as industrial proteins and enzymes. The less processing required for a formulation, the more cost-effective the manufacturing. Thus, direct addition of the plant part containing the enzyme of interest saves money on production and increases the margin for the producer. Direct addition would be particularly useful for industrial enzymes that accumulate in dry seed, such as corn, where stability is ensured in the seed until such time as it is used (Howard et al. 2011).

An additional advantage is when current agricultural crops are used as plant hosts; their production and processing are well established and usually inexpensive. As an example, corn requires few inputs other than nitrogen if grown in the corn belt. Dry mill processing is very well established on a volume basis, and every fraction of the whole or milled corn has a market. If value can be added to one of the lower value coproducts, for example, by putting a high-value protein in the germ (Hood et al. 2007), then an advantage is gained in increasing the value of this coproduct of the corn-to-ethanol industry.

Scaling up production of proteins from crops is also advantageous over animal or microbial systems. For crops, scale-up involves planting and harvesting more acres and does not require additional capital investment in physical infrastructure. The only capital investment involves planting and harvesting equipment, which, although somewhat expensive, does not require the level of investment required for scaling up microbial or animal systems. Thus, high-volume production can be achieved relatively easily.

1.3 Issues for Commercialization

Intellectual property for the specific gene and its expression in a plant host is only one part of the legal landscape for commercializing products using the plant production platform. Plant-enabling methods have been developed over many years with many companies and university laboratories participating in the

platform. Thus, a plethora of patents surround the technology and are often barriers to entry for commercialization of products from genetically engineered plants. During the development of potential products, it is critical to be aware of the technology pieces that are utilized to ensure freedom to operate on the pieces. Licenses for technology can sometimes burden the developer with high royalty fees, pushing the products' costs to a price greater than they are worth.

1.3.1 Regulatory Issues and Public Acceptance

1.3.1.1 Europe

The European Food Safety Authority (EFSA) is a European Union (EU) agency mandated to evaluate the risks of all transgenic crops based on scientific evidence. This evidence is evaluated by a panel of experts, and testing is carried out at an EU reference laboratory. As such, EFSA is best placed to advise individual Member States and the EU as a whole on safety issues (Sabalza et al. 2011). EU legislation for the approval of GE crops (Directive 2008/27/EC and Regulation EC 1829/2003) is the most onerous and restrictive in the world. Regulatory compliance for a new crop with first-generation simple agronomic traits can cost up to €11 million (~US$15 million) and requires a dedicated legal team working for many years (Kalaitzandonakes et al. 2007).

The EU regulatory approach is precautionary, process-based, and includes mandatory labeling and traceability requirements (Ramessar et al. 2008). The approach has been described in detail in a recent review (Sparrow et al. 2013). Briefly, EU legislation is adopted through a system of interactions between the three main EU institutions: the European Parliament, the Council of the European Union, and the European Commission (Sparrow et al. 2013). The EFSA published guidance notes in 2009 on the risk assessment of genetically modified plants used for nonfood or non-feed purposes (EFSAPanel 2009) including molecular pharming applications. The European Medicines Agency (EMEA) that oversees the assessment of biopharmaceuticals and vaccines published guidance notes in 2006 on the "quality of biological active substances produced by stable transgene expression in higher plants" (EMEA 2008), which looks at such issues.

More recently a further requirement was imposed on all transgenic plants, including those for molecular pharming applications. The European Commission mandated a compulsory 90-day animal feeding trial and, to make matters even more complicated, is considering extending that to a 2-year trial based on the now-discredited article by Seralini et al. (Seralini et al. 2012; Arjó et al. 2013). The scientific community as well as regulators themselves questioned the validity of such whole food-based animal trials (Kuiper et al. 2013).

Once authorization has been received, farmers must ensure that they comply with the conditions laid down by the authorities in their Member State and/or local region, often finding that illegal national or regional bans on GM agriculture have

been imposed. Farmers must abide by the coexistence measures that have been implemented in each Member State or region, and the complexity of these regulations and their strict implementation often means that it is impossible to comply. The four major obstacles to GM agriculture in the EU post-authorization are:

1. Public field registers showing the location of commercially grown GM crops are compulsory in almost all Member States and tend to discourage farmers from adopting GM agriculture because of the threat of vandalism by activists.
2. Six Member States use a "safeguard clause" nominally based on environmental or health concerns, to implement national cultivation bans for approved GM crops (Austria, France, Germany, Greece, Luxemburg, and Hungary).
3. Stringent coexistence measures have been implemented in Belgium, the Czech Republic, Germany, Hungary, Portugal, Romania, and Slovakia, which make it impossible to grow GM crops without risking litigation from the surrounding farms.
4. The negative publicity surrounding GM agriculture in Europe, which means farmers are ostracized and intimidated directly or indirectly.

The public in Europe has adopted a predominantly anti-GM stance, which is fueled by politicians and media eager to exploit public sentiment. This vicious cycle also shows no sign of going away any time soon (Farre et al. 2011). As discussed above the rules governing the commercial cultivation of GM crops in Europe are obstructive and arbitrary, making it virtually impossible for a farmer to make an independent decision to adopt the technology on his/her land even if the crop in question has been approved (Ramessar et al. 2010).

Across Europe the political viewpoint of cultivating GM crops is far from harmonious, with a number of Member States banning such cultivation (http://www.greenbiotech.org) (Ramessar et al. 2008, 2009, 2010; Sabalza et al. 2011). Given the state of play surrounding the cultivation of agricultural GM crops, it is unlikely that we will see a pharmaceutical crop grown commercially in Europe any time soon (Masip et al. 2013; Sparrow et al. 2013).

1.3.1.2 United States

Regulations in the USA for transgenic plants are set by the United States Department of Agriculture (USDA), the Food and Drug Administration (FDA), and the Environmental Protection Agency (EPA). The regulatory framework is complex and expensive with a lack of standardization for data collection and analysis (Hood et al. 2012). The framework is somewhat coordinated in that each agency is responsible for specific types of approvals—USDA for plant pests, FDA for food and feed issues, and EPA for pesticides, although sometimes the lines overlap or are blurred. A recent review describes the legislation and several case studies that apply the standards as they currently stand in the USA (Sparrow et al. 2013). To facilitate the process, particularly for small and specialty crop developers, a basic road map

should be created from which a specific regulatory path can be planned and implemented (Hood et al. 2012).

Public acceptance in the USA is much less of an issue than in Europe. Although anti-GMO groups are active in the USA, their impact has waned over the years. The success of genetically engineered crops has been good, showing higher yields and fewer pesticide or herbicide inputs. The vast majority of corn and soybeans in the USA are produced from GE crops and occur in many processed foods. Thus, even though some resistance occurs against GE plants in the press, the basic fact is that most citizens are consuming GE foods on a daily basis without incident. Indeed, each of the crops was subjected to a vast array of safety studies that were reviewed not only by the USDA APHIS but also by the FDA to ensure human safety. Miller (2011, 2012) published some editorial opinion pieces recently on the status of GE crops worldwide and received a great deal of criticism. However, the facts are correct and supported by such groups as the Grocery Manufacturers Association (http://www.gmaonline.org/news-events/newsroom/gma-commends-ama-action-in-support-of-continued-use-of-genetically-engineer/).

1.4 The Case Studies

Several reviews of plant-produced proteins have been written over the last several years (Fischer et al. 2004; Stoger et al. 2005; Streatfield 2007; Daniell et al. 2009; Egelkrout et al. 2012). Each of these reviews describes issues concerning expression, different product categories, and advantages of different plant systems. Plant biotechnology and gene transfer have been practiced as a technology since the early 1980s, and the vast majority of products commercialized have been input traits that assist with production, e.g., insect and herbicide resistance (Castle et al. 2006; Fraley 2009).

In this volume, the focus is on products from plants that either have been commercialized or that are near commercialization. We have chosen protein products that illustrate the promise of the system, for example, highly purified proteins without concerns over animal pathogen contaminants and directly delivered proteins—orally delivered vaccines or minimally processed industrial products. The promise of plant-made recombinant proteins was first realized in 1997 with the introduction of avidin and β-glucuronidase. Recently, pharmaceuticals (PMP) and vaccines as well as industrial proteins (PMIP) have just recently been consummated with the introduction of Syngenta's Enogen corn that contains amylase for the starch to ethanol application (Pollack 2011) and Protalix and Pfizer's glucocerebrosidase for enzyme replacement therapy (Aviezer et al. 2009; Ratner 2010). The products described in these chapters do not represent all the work that has been done in transgenic plants but do represent several that have been moved into or near commercialization.

Hood and Howard describe development of avidin in corn seed, originally transformed in as a potential candidate for insect resistance. Although the insect resistance trait was not commercialized, avidin was subsequently purified from seed and sold. This product was a key achievement for the plant manufacturing industry as the first protein sold from transgenic plants (Hood et al. 1997) (Sigma Chemical Co. A8706) and set the stage for this platform (Chap. 2). Although the avidin market is small, its importance cannot be overstated since it was the demonstration product for the technology. The main application of this protein is as a research reagent that allowed quick market entry.

Other types of products such as vaccines and pharmaceuticals were also in development concurrently but had much longer timelines for market entry. Fischer et al. (Chap. 3) describe multiple therapeutics that include antibodies for several applications manufactured in plant production systems. These therapeutics are produced by a number of different platform technologies, and the issues for their commercialization are discussed in the context of these new products.

Krishnan and Woodard (Chap. 4) describe the development of recombinant trypsin from the maize seed production system. This product is sold under the trade name TrypZean™ and is currently used for research and for processing of therapeutic proteins. One of the largest applications of trypsin is the maturation of recombinant insulin, and the plant-derived protein could be a great improvement in this process since it is animal product-free and would not pose threats to the drug's use.

Aprotinin is manufactured in the transient tobacco system using an engineered tobacco mosaic virus vector (Chap. 5). It has major applications in surgery as a preventative for perioperative blood loss. The plant-made aprotinin is currently not approved for human use but has applications as a protease inhibitor in cell culture.

Vaccines are particularly well suited for plant production because of broad application and current need for a cold chain. Vaccines against a number of viruses have been developed using plant expression systems. Pandemic flus can threaten world health quickly and catastrophically. In order to address the need for rapid development of vaccines against urgent threats, Medicago Inc. established a platform technology that addressed surge capacity, speed, adaptability, and affordable cost per dose. The company developed a vaccine against the H1N1 flu virus in a transient tobacco expression system (Chap. 6) and showed efficacy in Phase I and Phase II clinical trials. Further development of the vaccine will be performed by Mitsubishi who recently acquired Medicago.

Malarial vaccines are extremely useful in tropical climates where mosquitoes are abundant. Streatfield et al. (Chap. 7) discuss the transient tobacco transformation system for the production of such a vaccine against the malarial parasite that is spread by the mosquito vector. Subunit vaccines using individual proteins have been difficult to develop because of the difficulty in expressing the individual antigens. The plant system has been particularly useful in this regard.

Transmissible gastroenteritis virus (TGEV) is a common pathogen of swine and is particularly dangerous to newborn piglets. Rajan (Chap. 8) describes the development of a subunit vaccine in corn seed that shows efficacy against the disease, particularly when delivered orally either through feed or colostrum from the sows. Although this highly efficacious and easily administered vaccine is available, it has not been adopted by the swine industry.

Many species and strains of rabies virus are known, posing a threat to human health worldwide, but particularly in developing countries. Loza-Rubio and Rojas-Anaya (Chap. 9) discuss the issues surrounding the development of a rabies vaccine based on the G-protein expressed in either corn seed or carrot roots. Both sources of the protein provided protection against the rabies virus in superinfected animals. These results are promising for the future of inoculation of wild animal populations to lower the load of infective viruses.

Newcastle disease virus is highly infective in avian species and can devastate poultry production in many countries. Gomez-Lim (Chap. 10) describes the development of plant-based vaccines against this virus using the corn/sorghum seed system for oral delivery or the tobacco system for injectable delivery. The ease of delivery of oral products would seem the preferred route and various issues to be overcome for this application are discussed.

Although several injectable vaccines for hepatitis B virus (HepB) are available, infection with this virus remains a world health problem. Hayden discusses the development and feeding trials of a plant-made oral vaccine from corn grain (Chap. 11). Oral vaccines have many advantages in that they have higher rates of dose compliance among susceptible populations. Using formulations of corn germ derived from transgenic plants expressing the S antigen, successful production of mucosal protective antibodies was achieved in mice.

Hood and Requesens (Chap. 12) describe the development of the industrial enzymes endo- and exo-cellulase in maize grain. These enzymes have applications in research, pulp processing, and biomass conversion. Early markets have been addressed with these products, and production lines have been established.

Finally, the sweet protein brazzein has been produced in maize grain. Fake and Howard (Chap. 13) describe the applications of this protein in various food-related industries and the effort to interest food companies in its use. Because the protein is a natural sweetener from an African fruit, it would be a logical substitution for such artificial sweeteners as acesulfame potassium or aspartame, particularly also because brazzein is about 1,000 times sweeter than sugar.

In the final chapter, the future of the plant-based production industry is discussed. Prospects are promising, but the major commercialization barrier is still overcoming the regulatory hurdles. Drs. Howard and Hood are pleased to present these case studies of plant-made proteins as a tribute to our colleague, Dr. Michael Horn.

References

Arjó G, Portero M, Piñol C, Viñas J, Matias-Guiu X, Capell T, Bartholomaeus A, Parrott W, Christou P (2013) Plurality of opinion, scientific discourse and pseudoscience: an in depth analysis of the Séralini et al. study claiming that Roundup™ Ready corn or the herbicide Roundup™ cause cancer in rats. Transgenic Res 22:255–267. http://parrottlab.uga.edu/parrottlab/Publications/Arjo-et-al-TRAG-2013.pdf

Aviezer D, Brill-Almon E, Shaaltiel Y, Hashmueli S, Bartfeld D, Mizrachi S, Liberman Y, Freeman A, Zimran A, Galun E (2009) A plant-derived recombinant human glucocerebrosidase enzyme—a preclinical and phase I investigation. PLoS One 4:e4792

Castle LA, Wu G, McElroy D (2006) Agricultural input traits: past, present and future. Curr Opin Biotechnol 17:105–112

Daniell H, Singh ND, Mason H, Streatfield SJ (2009) Plant-made vaccine antigens and biopharmaceuticals. Trends Plant Sci 14:669–679

EFSAPanel (2009) Scientific opinion on guidance for the risk assessment of genetically modified plants used for non-food or non-feed purposes. EFSA J 1164:1–42, E.F.S. Authority, ed (Parma, Italy: EFSA)

Egelkrout E, Rajan V, Howard JA (2012) Overproduction of recombinant proteins in plants. Plant Sci 184:83–101

EMEA (2008) Guideline on the quality of biological active substances produced by stable transgene expression in higher plants. In: E.M. Agency (ed) EMEA/CHMP/BWP/48316/2006. EMEA, London, pp 1–11

Farre G, Twyman RM, Zhu C, Capell T, Christou P (2011) Nutritionally enhanced crops and food security: scientific achievements versus political expediency. Curr Opin Biotechnol 22:245–251

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

Fraley RT (2009) Molecular genetic approaches to maize improvement–an introduction. In: Kriz AL, Larkins BA (eds) Molecular genetic approaches to maize improvement. Springer, Berlin, pp 3–6

Hayden CA, Streatfield SJ, Lamphear BJ, Fake GM, Keener TK, Walker JH, Clements JD, Turner DD, Tizard IR, Howard JA (2012) Bioencapsulation of the hepatitis B surface antigen and its use as an effective oral immunogen. Vaccine 30:2937–2942

Hood E, Witcher D, Maddock S, Meyer T, Baszczynski C et al (1997) Commercial production of avidin from transgenic maize: characterization of transformant, production, processing, extracting, and purification. Mol Breed 3:291–306

Hood EE, Love R, Lane J, Bray J, Clough R, Pappu K, Drees C, Hood KR, Yoon S, Ahmad A, Howard JA (2007) Subcellular targeting is a key condition for high-level accumulation of cellulase protein in transgenic maize seed. Plant Biotechnol J 5:709–719

Hood EE, Vicuna-Requesens D, Eversole KA (2012) Regulatory issues of biotechnologically improved plants. In: Altman A, Hasagawa PM (eds) Plant biotechnology and agriculture; prospects for the 21st century. Academic, London, pp 541–550

Howard JA, Hood E (2005) Bioindustrial and biopharmaceutical products produced in plants. Adv Agron 85:91–124

Howard J, Nikolov Z, Hood E (2011) Enzyme production systems for biomass conversion. In: Hood E, Nelson P, Powell R (eds) Plant biomass conversion. Wiley-Blackwell, Ames, IA, pp 227–253

Kalaitzandonakes N, Alston JM, Bradford KJ (2007) Compliance costs for regulatory approval of new biotech crops. Nat Biotechnol 25:509–511

Kuiper HA, Kok EJ, Davies HV (2013) New EU legislation for risk assessment of GM food: no scientific justification for mandatory animal feeding trials. Plant Biotechnol J 11:781–784

Lamphear BJ, Jilka JM, Kesl L, Welter M, Howard JA, Streatfield SJ (2004) A corn-based delivery system for animal vaccines: an oral transmissible gastroenteritis virus vaccine boosts lactogenic immunity in swine. Vaccine 22:2420–2424

Masip G, Sabalza M, Pérez-Massot E, Banakar R, Cebrian D, Twyman RM, Capell T, Albajes R, Christou P (2013) Paradoxical EU agricultural policies on genetically engineered crops. Trends Plant Sci 18:312–324

Mason HS, Warzecha H, Mor T, Arntzen CJ (2002) Edible plant vaccines: applications for prophylactic and therapeutic molecular medicine. Trends Mol Med 8:324–329

Miller H, Concko G (2011) The rush to condemn genetically modified crops. http://www.hoover.org/research/rush-condemn-genetically-modified-crops-0

Miller H (2012) NPRs bias against genetic engineering. http://www.forbes.com/sites/henrymiller/2012/02/01/nprs-bias-against-genetic-engineering/

Pollack A (2011) US approves corn modified for ethanol. The New York Times (February 11, B1)

Ramessar K, Capell T, Twyman RM, Quemada H, Christou P (2008) Trace and traceability—a call for regulatory harmony. Nat Biotechnol 26:975–978

Ramessar K, Capell T, Twyman RM, Quemada H, Christou P (2009) Calling the tunes on transgenic crops: the case for regulatory harmony. Mol Breed 23:99–112

Ramessar K, Capell T, Twyman RM, Christou P (2010) Going to ridiculous lengths—European coexistence regulations for GM crops. Nat Biotechnol 28:133

Ratner M (2010) Pfizer stakes a claim in plant cell-made biopharmaceuticals. Nat Biotechnol 28:107–108

Sabalza M, Miralpeix B, Twyman RM, Capell T, Christou P (2011) EU legitimizes GM crop exclusion zones. Nat Biotechnol 29:315–317

Séralini G-E, Clair E, Mesnage R, Gress S, Defarge N, Malatesta M, Hennequin D, de Vendômois JS (2012) Long term toxicity of a Roundup herbicide and a Roundup-tolerant genetically modified maize. Food Chem Toxicol 50:4221–4231

Sparrow P, Broer I, Hood EE, Eversole K, Hartung F, Schiemann J (2013) Risk assessment and regulation of molecular farming-a comparison between Europe and US. Curr Pharm Des 19:5513–5530

Stoger E, Ma JKC, Fischer R, Christou P (2005) Sowing the seeds of success: pharmaceutical proteins from plants. Curr Opin Biotechnol 16:167–173

Streatfield S (2007) Approaches to achieve high-level heterologous protein production in plants. Plant Biotechnol J 5:2–15

Tacket CO, Mason HS, Losonsky G, Clements JD, Levine MM, Arntzen CJ (1998) Immunogenicity in humans of a recombinant bacterial antigen delivered in a transgenic potato. Nat Med 4:607–609

Part I
Highly Purified Proteins

Chapter 2
Commercial Plant-Produced Recombinant Avidin

Elizabeth E. Hood and John A. Howard

2.1 Introduction to the Protein Product

Chicken egg white avidin was the first recombinant protein product manufactured for sale in a transgenic plant. Prior to its commercialization, there were many questions as to the validity of using plants as a platform to produce recombinant proteins: doubts were raised as to the ability of plants to express animal or microbial proteins, the ability to obtain proper processing and glycosylation, and the ability to extract and purify these proteins in an economical manner. Therefore, while avidin has modest economic value, it served as the model to launch this approach for a number of other recombinant proteins.

Avidin (C.A.S.: 1405-69-2) is a glycoprotein found in avian, reptilian, and amphibian eggs and is used commercially as a diagnostic reagent. It was first isolated from chicken egg white and named "avidin" in the 1940s (Thompson et al. 1941). The protein avidin comprises four identical subunits, each 128 amino acids long, the amino acid sequence of which was published in 1971 (DeLange and Huang 1971). The carbohydrate moiety is composed of four glucosamine and five mannose residues and is attached to Asn-17 of each subunit (DeLange and Huang 1971). The cDNA of the chicken oviduct *avidin* gene was identified (Gope et al. 1987) and a genomic clone was isolated (Keinanen et al. 1988). They (Keinanen et al. 1988) also reported on a family of closely related *avidin* genes from chicken.

Avidin binds the vitamin biotin with high affinity. Each of the four subunits in the homotetramer binds one biotin molecule. The dissociation constant of the avidin–biotin complex was determined to be 10^{-15} (Green 1963), exhibiting the

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highest known affinity in nature between a ligand and a protein (Livnah et al. 1993). The binding of avidin to biotin is responsible for its commercial value, since it allows for detection of proteins and nucleic acid molecules incorporating biotin. Avidin or avidin subunits can also be used for affinity purification of biotinylated molecules (Berger and Wood 1975; Green and Toms 1973). In nature biotin functions as a cofactor with many enzymes in vivo. Because avidin binds strongly to biotin, it can act as a defense agent against microbial pathogens that are sensitive to biotin levels (Wallen et al. 1995). A second biotin-binding protein is bacterial streptavidin. Although these two proteins show similar activity and tertiary structure, their amino acid sequences are only 30 % identical and are likely not derived from the same ancestral source (Laitinen et al. 2006).

Scientists at Pioneer Hi-Bred International noticed that avidin could inhibit growth in some insects by interfering with their digestion. Transgenic maize plants expressing the chicken *avidin* gene were generated to test it as an insecticidal reagent incorporated into maize leaves and roots (Hood et al. 1997). This observation was later followed up and shown to be very effective to prevent postharvest insect damage while not interfering with metabolism in mammals (Kramer et al. 2000). The transgenic maize plants had a secondary phenotype in that they could confer male sterility and have been suggested as a containment mechanism for transgenic traits in the field (Albertsen et al. 1999).

The primary source for commercial production of avidin is chicken egg white, although the recombinant form is also available (Sigma Chemical Co. A8706). The manufacture of purified avidin protein using a plant source as an alternative to eggs provides benefits such as the absence of animal viruses. Plant-produced avidin provided answers to many of the basic questions about plant-expressed proteins and what is critical for commercialization, providing conditions that are in use today.

2.2 Description of the Systems Used to Produce the Protein

2.2.1 Theoretical Advantages of the Plant Process over Other Technologies

Avidin is usually purified from egg whites (http://www.mastbio.co.kr/root/product/life/ps/gradiflow/pdf/MB-10-Puri-HighlyBasicProteinsAvidinandLysozyme.pdf), where it is present at a concentration of approximately 1.5 mg per egg. More recently, biologically active recombinant isoforms have been produced in several expression systems, including *Escherichia coli* (Airenne et al. 1994), *Picchia* (Zocchi et al. 2003), and baculovirus-infected cells (Airenne et al. 1997). A huge number of variants of avidin have been produced that have applications in various diagnostic and purification kits (Laitinen et al. 2006).

The advantages of a plant recombinant system over the others currently used are that: (1) scale-up is more economical in a plant system due to less expensive substrates (corn grain versus eggs) and greater biomass availability, (2) co-purification of animal pathogens is avoided in a plant system, and (3) if expression is directed to seed, it provides a natural storage system for long duration without degradation.

2.2.2 Past Efforts in Plants

A number of laboratories have experimented with expressing avidin in plants, primarily for its insecticidal properties (Murray et al. 2002; Lichtfouse et al. 2010; Burgess et al. 2002; Markwick et al. 2003; Murray et al. 2010; Masarik et al. 2003). In many cases, the transgenic plants were insect resistant reaching the goal of the project.

2.2.3 Bench Marks of What Is/Was Needed to Commercialize the Product in This System

Most of the initial work with avidin expression in different plants was not designed to overproduce the protein for purification and sale. The maize seed production system, on the other hand, was suitable for the production of the protein for sale as a purified or partially purified product primarily for use in diagnostic kits. High-level expression is required for cost-effective production in the plant system to meet commercial targets. Assuming that the competitive production system is from egg whites, one dozen eggs would produce about 18 mg of avidin for a cost of about $2 for the raw materials. Eighteen mg of recombinant protein from corn seed expressing the protein at 1 % of total soluble protein would require approximately 200 g of grain. At today's high price of $7 per bushel (25 kg), this grain would cost ~$0.06. One percent of TSP has been achieved for multiple proteins in corn seed, and avidin levels as high as 40–50 % of TSP in some selected lines have been obtained. Clearly, the corn system offers economic advantages over the egg system as it relates to the cost of raw materials. In addition, higher concentration of avidin in the biomass leads to a lower cost of purification.

Because proteins produced from plants were new to the market, quality assessment of the product had to be performed to understand the impurities in the product and to build a certificate of analysis, a quality control protocol, and a Material Safety Data Sheet (MSDS) for the product. Each of these was developed for this new product for Sigma Aldrich Chemical Co., which is still the vendor for the product. Characteristics of the protein and product are described below.

2.3 Technical Progress

2.3.1 What Was Achieved?

Many technical tools that were sought after in the mid-1990s are the same today for expression of foreign genes in plants. These include use of a strong promoter, use of an intron particularly for monocot expression, recognition of the need for codon usage that is compatible with the host species, avoidance of toxicity, and targeting the protein to specific subcellular locations that induce maximum expression of the protein (Streatfield 2007). Indeed, each of these molecular parameters was utilized for avidin.

Avidin in maize seed was first produced over 16 years ago (Hood et al. 1997). The molecular technology available at the time was much less sophisticated than technology available today. The gene was synthesized with maize codon usage bias and fused with the barley alpha amylase signal sequence (BAASS) (Rogers 1985), also synthesized with maize codons. Each of the genes/fragments was synthesized as short, overlapping, complementary oligonucleotides with restriction enzyme sites engineered onto the ends and ligated after digestion. All movement between cloning vectors was done with restriction enzyme digestion and ligation. The expression cassette with the constitutive maize ubiquitin promoter (Christensen et al. 1992) and the *pinII* terminator (An et al. 1989) was built separately from the herbicide selection vector for co-bombardment of maize callus tissue. Selection was on the herbicide, bialaphos, using the *bar* gene (White et al. 1990) driven by the CaMV 35S promoter. At that time, biolistic transformation was the most efficient way to introduce genes into corn (USP#5,489,520).

2.3.2 What Expected or Unexpected Hurdles Were Overcome to Reach the Target?

Transgenic events that were resistant to bialaphos and contained the avidin gene as identified by PCR were recovered from transformations. Plants were regenerated from these events; they produced ears in a greenhouse and were pollinated with a proprietary inbred line (Pioneer Hi-Bred PHN46). The highest expressing event was screened by DNA blot hybridization for copy number and insertion sites (Hood et al. 1997). It appeared that three to five insertions were present in this event for both the *avidin* and *bar* genes. When T1 seed was planted for seed increases, the T2 generation plants were no longer resistant to the herbicide. Thus, another method of screening for the segregating (transgenic versus non-transgenic) plants was required. Initially, PCR was performed to track the presence of the *avidin* gene. Observations of the plants in the field revealed that male sterility was present among them at a high percentage. When the PCR results were compared to the

Table 2.1 Increases in inbred germplasm and avidin during several back cross generations of breeding and selection [derived from Hood (2004)]

Year	Generation of see post tissue culture	% Inbred germplasm	Avidin as % DW of grain
1995	T1	50	0.01
1996	T2	75	0.02
1997	T3	87.5	0.05
1998	T4	93.75	0.1
1999	T5	96.88	0.3
2000	T6	98.44	0.7
2001	T7	99.2	1.0

male sterility phenotype, it was discovered that these traits co-segregated to a high degree (97.5 %). Thus, in future generations, transgenic plants expressing the avidin gene were selected by their male sterile phenotype. Although this phenotype is useful for sequestration of the transgenic trait in the environment, it inhibits the ability to recover self-pollinated, homozygous lines.

The Hi-II tissue culture genotype (Armstrong et al. 1991) does not produce well in the field and is highly susceptible to insects and pathogens. Thus, when transformation is performed with the Hi-II line, the resulting events must be back-crossed into elite inbred lines to allow production lines to be established. During back-crossing, it was discovered that increases in transgene-encoded protein could also be recovered in addition to improved agronomic characteristics (Hood et al. 2012). Prior to this point, it was assumed by most scientists that the expression level in T1 seeds would be the same for future generations. However, for maize, introgression into elite lines with selections for expression at each generation led to much higher levels of target protein accumulation. This initial observation has held true for all foreign proteins that we have expressed in corn although they accumulate in seed to greater or lesser amounts (Streatfield et al. 2002; Woodard et al. 2003; Hood et al. 2003, 2012). For avidin, we recovered up to 1 % of dry weight of grain in later generations (Table 2.1) (Hood 2004). Some of this improvement is due to improved germplasm and some is due to more stringent selection of transgenic lines using the male sterility trait to prevent mixing of avidin and non-avidin plants. However, the mechanism(s) driving the improvement in accumulation is unknown. Modern genomic and transcriptomic techniques however should allow a more satisfactory explanation in the near future (Teoh et al. 2013).

The corn-produced avidin was functionally equivalent to the egg-derived protein (Table 2.2). The only obvious physical difference was that the corn protein had slightly less glycosylation—the deglycosylated maize-derived proteins showed the same molecular weight as the native apoprotein (Hood et al. 1997). Binding of the complex protein to biotin, the N-terminal protein sequence, and the pI was the same for protein from either source. These characteristics allow its direct substitution into assay kits from a functionality standpoint.

Because avidin was one of the first highly expressed plant-produced proteins, it was used for many demonstration and pilot studies. One such study was to discover the characteristics of a protein fed to animals (Fig. 2.1). Were antibodies produced?

Table 2.2 Comparison of biochemical properties of native egg avidin and corn-derived recombinant avidin

Biochemical properties	Egg white avidin	Recombinant avidin
Binding stoichiometry	Binds one biotin per subunit	Binds one biotin per subunit
pI	10	10
KI	3.16 μM	3.34 μM
Antigenic similarity	Identical	Identical
Glycosylated	Yes	Yes
N-terminal sequence	Identical	Identical
Molecular weight apoprotein	12,500 kDa	12,500 kDa

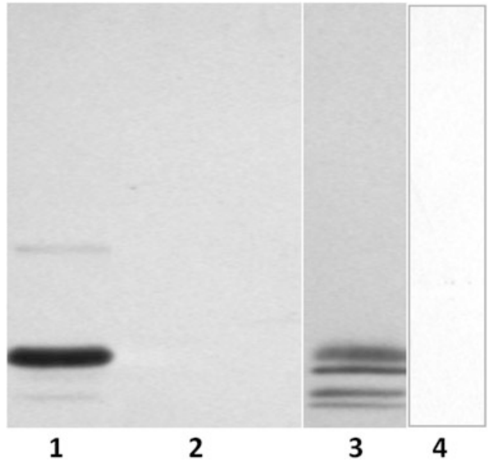

Fig. 2.1 Western blot of feces extracts from mice fed various diets. Total protein was separated on a 12 % SDS polyacrylamide gel and proteins were blotted onto a PVDF membrane (Millipore). Detection of avidin was with an anti-avidin antibody and a secondary antibody conjugated to alkaline phosphatase (AP). AP was detected with a chemiluminescent substrate. Lanes: 1: 50 mg standard avidin; 2: control corn diet; 3: avidin corn diet; 4: mouse chow with 50 mg pure avidin added

If so, what types of antibodies were they? What was the fate of the protein after being fed to animals? These, and other questions, were addressed in a Master of Science thesis study at Texas A&M University (Bailey 2000).

"Avidin corn meal was successfully used to stimulate both serum and mucosal immune responses when fed as the sole diet of mice for several days" (Bailey 2000). In these early studies, seven doses of formulated corn meal were used to stimulate a mucosal response, while nine doses were required for a serum response. Corn was fed as the sole diet, and the doses administered through various feeding regimens of corn meal plus or minus avidin. One of the most interesting outcomes of this study was that the protein survived in the gut after ingestion only when the protein was encapsulated into the grain matrix. Doses of control avidin that comprised purified protein added to the mouse diet were completely degraded in

the gut. This outcome has implications for the quality of antigen presentation to the immune system when fed orally to an animal and was the basis to explore orally administered vaccines (discussed in later chapters in this book).

2.4 Nontechnical Hurdles

2.4.1 Production, Regulatory, Public Perception, Intellectual Property

Although avidin is used in multiple diagnostic products for multiple markets, the production volumes are relatively small: in the order of hundreds of kilograms per year, rather than tons. At the average concentration of 1.5 mg of avidin per egg, a dozen eggs would only produce 18 mg, an amount available from 3.5 g of corn (Fig. 2.2). Indeed, over 800 kg of eggs would be required to produce 20 g of avidin (Hood et al. 1997). Currently, the concentration of avidin in corn seed is up to an average of approximately 0.5 % of dry weight; thus, 4 kg of grain would be required to yield the equivalent 20 g of avidin. Corn grain weighs 25 kg per bushel, and 20 g of avidin could be produced from about 1/6 of a bushel of corn. At 2013 prices, that would be about $1 worth of corn. Even if one triples the price for small volumes and growth under permit, the cost would only be about $3 for the raw materials for protein production. Clearly, this is an advantage over production costs of eggs.

In addition to the cost of raw material, the processing to a highly purified product is usually the most expensive part of the final product. One of the most critical cost factors in this regard is the concentration of the protein in the biomass that is to be used in extraction. Higher concentrations lead to lower unit costs of extraction and usually require less effort in purification as well. In this case, >tenfold higher concentrations in the initial maize biomass can drastically reduce downstream purification cost. Furthermore, as this protein accumulates predominantly in the germ of the kernel, the routine operation of separating the germ from the endosperm as performed in dry milling operations can result in another tenfold reduction in biomass and further reduce downstream cost. Cost models have been created using these factors that have been discussed previously (Howard et al. 2011). An additional advantage is the long-term stability of the product in the grain allowing storage of the raw material for months to years (Kusnadi et al. 1998).

The male sterility trait of the avidin corn does not allow for making homozygous lines. Thus, the trait segregates at every generation, forcing selection of the transgenics from the null segregants for production. Loss of the herbicide resistance trait early on makes production challenging because selection of the transgenic plants cannot be done with the herbicide. Thus, visual scoring is now the only technique with which selection can be done, forcing labor-intensive selection. Fortunately, the amount of protein required for production is low, and this type of production scheme is not prohibitively expensive. One acre of avidin at 0.5 % of dry weight

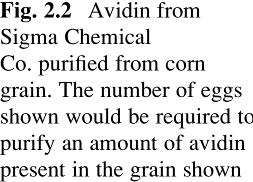

Fig. 2.2 Avidin from Sigma Chemical Co. purified from corn grain. The number of eggs shown would be required to purify an amount of avidin present in the grain shown

and 160 bushels per acre would yield 20 kg of protein, easily within the production quantities required.

Production of transgenic seed crops for reagent chemicals is done under permit because of the small volume market opportunity. Thus, nonregulated status was not sought and likely will not be necessary to make a profit on the products. These product volumes do not impact the food versus nonfood debate of using plants for purposes other than for food because of their small volume production. At 20 kg per acre, the entire annual product volume of avidin would require fewer than 100 acres. When considering the total volume of corn production, i.e., approximately 100 million acres, then 100 acres for a specialty product have no significant impact.

Even using the best containment precautions, there is always the concern that inadvertent exposure could occur due to some unforeseen event. This is usually thought of as unwanted pollination. In this case, however, this is highly unlikely since the recombinant avidin causes male sterility. Pollen drift is not the only method of exposure, however, and like all other non-plant production systems, contamination of the food chain in any number of ways is considered when setting containment guidelines. The maize production system offers another safeguard in that corn is cooked prior to human consumption which will completely inactivate avidin. This is most evident in that we consume eggs routinely without any adverse effect as long as they are cooked.

2.5 Conclusions

Twenty years ago, the concept of proteins produced in plants was novel. The early successes were important to demonstrate that the technology works. Production of avidin in transgenic maize fulfilled that demonstration. Plant-produced avidin was also used to demonstrate that orally fed proteins could induce circulating and mucosal antibodies in mice. The fact that small amounts of the fed protein, in this

case avidin, survived the gut was an unexpected discovery, but helped to explain why other orally fed purified proteins were not successful vaccines—the protein had to be part of dietary fiber to be protected long enough in the gut to induce a response. The corn containing avidin proved to be resistant to grain storage insects (Kramer et al. 2000). Of additional interest, however, is the male sterile phenotype induced by the avidin gene expressed from the constitutive ubiquitin promoter. Avidin as the first plant-produced protein sold set the stage and debunked pessimism about plant-produced proteins.

2.6 Future Directions

The worldwide market for avidin as a component of diagnostic kits is in the kilogram range. Thus, using the corn seed production system, this market could easily be filled from a few acres of production. More lucrative applications of avidin would be as an inducer of male sterility or insect resistance. These latter uses of the avidin trait would have utility in agriculture, but would require development by a seed company. Continued reagent sales and potential application in diagnostic kits are the most likely market outcomes.

References

Airenne KJ, Sarkkinen P, Punnonen E-L, Kulomaa MS (1994) Production of recombinant avidin in Escherichia coli. Gene 144(1):75–80, http://dx.doi.org/10.1016/0378-1119(94)90206-2

Airenne KJ, Oker-Blom C, Marjomäki VS, Bayer EA, Wilchek M, Kulomaa MS (1997) Production of biologically active recombinant avidin in baculovirus-infected insect cells. Protein Expr Purif 9(1):100–108, http://dx.doi.org/10.1006/prep.1996.0660

Albertsen MC, Howard JA, Maddock S (1999) Induction of male sterility in plants by expression of high levels of avidin. USA Patent

An G, Mitra A, Choi HK, Costa MA, An K, Thornburg RW, Ryan CA (1989) Functional analysis of the 3[prime] control region of the potato wound-inducible proteinase inhibitor II gene. Plant Cell 1(1):115–122. doi:10.1105/tpc.1.1.115

Armstrong C, Green C, Phillips R (1991) Development and availability of germplasm with high type II culture formation response. Maize Genet Coop News Lett 65:92–93

Bailey M (2000) A model system for edible vaccination using recombinant avidin produced in corn seed. Texas A&M University, College Station, TX

Berger M, Wood HG (1975) Purification of the subunits of transcarboxylase by affinity chromatography on avidin-sepharose. J Biol Chem 250(3):927–933

Burgess EJ, Malone L, Christeller J, Lester M, Murray C, Philip B, Phung M, Tregidga E (2002) Avidin expressed in transgenic tobacco leaves confers resistance to two noctuid pests, helicoverpa armigera and spodoptera litura. Transgenic Res 11(2):185–198

Christensen A, Sharrock R, Quail P (1992) Maize polyubiquitin genes: structure, thermal perturbation of expression and transcript splicing, and promoter activity following transfer to protoplasts by electroporation. Plant Mol Biol 18:810–812

DeLange RJ, Huang TS (1971) Egg white avidin. 3. Sequence of the 78-residue middle cyanogen bromide peptide. Complete amino acid sequence of the protein subunit. J Biol Chem 246 (3):698–709

Gope ML, Keinanen RA, Kristo PA, Conneely OM, Beattie WG, Zarucki-Schulz T, O'Malley BW, Kulomaa MS (1987) Molecular cloning of the chicken avidin cDNA. Nucleic Acids Res 15(8):3595–3606

Green NM (1963) Avidin. 3. The nature of the biotin-binding site. Biochem J 89:599–609

Green NM, Toms EJ (1973) The properties of subunits of avidin coupled to sepharose. Biochem J 133(4):687–700

Hood EE (2004) Bioindustrial and biopharmaceutical products from plants. In: New directions for a diverse planet: proceedings for the 4th international crop science congress, 26 September–1 October 2004, The Regional Institute Ltd, Brisbane, Australia

Hood E, Witcher D, Maddock S, Meyer T, Baszczynski C et al (1997) Commercial production of avidin from transgenic maize: characterization of transformant, production, processing, extracting, and purification. Mol Breed 3:291–306

Hood EE, Bailey MR, Beifuss K, Magallanes-Lundback M, Horn ME, Callaway E, Drees C, Delaney DE, Clough R, Howard JA (2003) Criteria for high-level expression of a fungal laccase gene in transgenic maize. Plant Biotechnol J 1(2):129–140. doi:10.1046/j.1467-7652.2003.00014.x, PBI014 [pii]

Hood EE, Devaiah SP, Fake G, Egelkrout E, Teoh K, Requesens DV, Hayden C, Hood KR, Pappu KM, Carroll J, Howard JA (2012) Manipulating corn germplasm to increase recombinant protein accumulation. Plant Biotechnol J 10:20–30. doi:10.1111/j.1467-7652.2011.00627.x

Howard JA, Nikolov Z, Hood EE (2011) Enzyme production systems for biomass conversion. In: Hood EE, Nelson P, Powell R (eds) Plant biomass conversion. Wiley, Ames, IA, pp 227–253, http://dx.doi.org/10.1002/9780470959138.ch10

Keinanen RA, Laukkanen ML, Kulomaa MS (1988) Molecular cloning of three structurally related genes for chicken avidin. J Steroid Biochem 30(1–6):17–21

Kramer K, Morgan T, Throne J, Dowell F, Bailey M, Howard J (2000) Transgenic avidin maize is resistant to storage insect pests. Nat Biotechnol 18:670–674

Kusnadi AR, Hood EE, Witcher DR, Howard JA, Nikolov ZL (1998) Production and purification of two recombinant proteins from transgenic corn. Biotechnol Prog 14(1):149–155

Laitinen OH, Hytonen VP, Nordlund HR, Kulomaa MS (2006) Genetically engineered avidins and streptavidins. Cell Mol Life Sci 63(24):2992–3017. doi:10.1007/s00018-006-6288-z

Lichtfouse E, Martin H, Burgess EJ, Masarik M, Kramer K, Beklova M, Adam V, Kizek R (2010) Avidin and plant biotechnology to control pests. In: Engineering G (ed) Biofertilisation, soil quality and organic farming, vol 4, Sustainable agriculture reviews. Springer, Netherlands, pp 1–21

Livnah O, Bayer EA, Wilchek M, Sussman JL (1993) Three-dimensional structures of avidin and the avidin-biotin complex. Proc Natl Acad Sci USA 90(11):5076–5080

Markwick N, Docherty L, Phung M, Lester M, Murray C, Yao J-L, Mitra D, Cohen D, Beuning L, Kutty-Amma S, Christeller J (2003) Transgenic tobacco and apple plants expressing biotin-binding proteins are resistant to two cosmopolitan insect pests, potato tuber moth and lightbrown apple moth, respectively. Transgenic Res 12(6):671–681

Masarik M, Kizek R, Kramer K, Billova S, Brazdova M, Vacek J, Baley M, Jelen F, Howard J (2003) Application of avidin-biotin technology transfer stripping square-wave voltammetry for detection of DNA hybridization and avidin in transgenic avidin maize. Anal Chem 75:2663–2669

Murray C, Sutherland P, Phung M, Lester M, Marshall R, Christeller J (2002) Expression of biotin-binding proteins, avidin and streptavidin, in plant tissues using plant vacuolar targeting sequences. Transgenic Res 11(2):199–214

Murray C, Markwick N, Kaji R, Poulton J, Martin H, Christeller J (2010) Expression of various biotin-binding proteins in transgenic tobacco confers resistance to potato tuber moth, Phthorimaea operculella (Zeller) (fam. Gelechiidae). Transgenic Res 19(6):1041–1051

Rogers JC (1985) Two barley alpha-amylase gene families are regulated differently in aleurone cells. J Biol Chem 260(6):3731–3738

Streatfield S (2007) Approaches to achieve high-level heterologous protein production in plants. Plant Biotechnol J 5:2–15

Streatfield S, Mayor J, Barker D, Brooks C, Lamphear B, Woodard S, Beifuss K, Vicuna D, Massey L, Horn M, Delaney D, Nikolov Z, Hood E, Jilka J, Howard J (2002) Development of an edible subunit vaccine in corn against enterotoxigenic strains of Escherichia coli. In Vitro Cell Dev Biol Plant 38(1):11–17. doi:10.1079/ivp2001247

Teoh KT, Requesens DV, Devaiah SP, Johnson D, Huang X, Howard JA, Hood EE (2013) Transcriptome analysis of embryo maturation in maize. BMC Plant Biol 13(1):19

Thompson RC, Eakin RE, Williams RJ (1941) The extraction of biotin from tissues. Science 94 (2451):589–590. doi:10.1126/science.94.2451.589

Wallen MJ, Laukkanen MO, Kulomaa MS (1995) Cloning and sequencing of the chicken egg-white avidin-encoding gene and its relationship with the avidin-related genes Avr1-Avr5. Gene 161(2):205–209

White J, Chang SY, Bibb MJ, Bibb MJ (1990) A cassette containing the bar gene of Streptomyces hygroscopicus: a selectable marker for plant transformation. Nucleic Acids Res 18:1062

Woodard S, Mayor J, Bailey M, Barker D, Love R, Lane J, Delaney D, McComas-Wagner J, Mallubhotla H, Hood E (2003) Maize (Zea mays)-derived bovine trypsin: characterization of the first large-scale, commercial protein product from transgenic plants. Biotechnol Appl Biochem 38(2):123–130

Zocchi A, Marya Jobé A, Neuhaus J-M, Ward TR (2003) Expression and purification of a recombinant avidin with a lowered isoelectric point in Pichia pastoris. Protein Expr Purif 32 (2):167–174, http://dx.doi.org/10.1016/j.pep.2003.09.001

Chapter 3
Molecular Farming in Plants: The Long Road to the Market

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3.1 Introduction

3.1.1 The History of Molecular Farming

The first recombinant proteins were expressed in plants in the early 1980s concurrent with the development of efficient transformation protocols based on *Agrobacterium tumefaciens*. In such cases, the proteins were the enzymatic products of bacterial selection markers, such as *npt*II encoding neomycin phosphotransferase (Bevan et al. 1983). Subsequently, plants were transformed with many different genes encoding products that conferred agronomic advantages such as herbicide tolerance and pest resistance, and only towards the end of the decade did scientists first address the possibility that plants could be used as a production system for recombinant proteins, i.e., with the ultimate objective of purifying the product rather than altering the phenotype of the plant (Hiatt et al. 1989). This was the birth of molecular farming (Ma et al. 2003).

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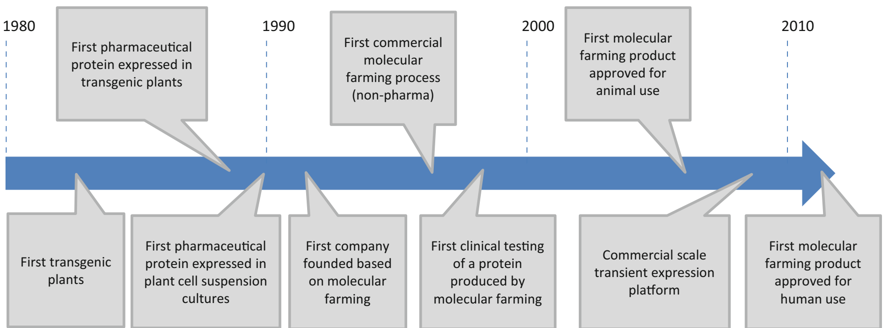

Fig. 3.1 Major landmarks in the commercial development of molecular farming

Following this technological breakthrough there was a 10-year gap before the first plant-derived recombinant proteins were produced commercially. This time was required to address technical barriers and make the process economically feasible. In every case, the resulting products were protein reagents for research or industrial enzymes (reviewed by Hood 2002). There was also considerable enthusiasm for plants to be used for the production of pharmaceutical proteins, a concept sometimes described as "molecular pharming." This was borne from the appreciation of several key advantages of plants compared to other production hosts, including the low cost of establishing and maintaining pharmaceutical crops compared to industrial-scale fermentation systems, the scalability of plants compared to fermenters, and the safety of plants compared to bacteria and mammalian cells, i.e., the lack of endotoxins and human pathogens that make most crops "generally regarded as safe" (Twyman et al. 2003). Based on this promise, many reports were published describing the production of pharmaceutical proteins in diverse plant-based systems; numerous small/medium enterprises (SMEs) were set up to capitalize on molecular farming, and large industry players began to take an interest (Twyman et al. 2005). However, it took until 2012 before the first pharmaceutical proteins from plants were approved for the market, and this long gap between proof of principle and commercial reality predominantly reflected the presence of regulatory barriers, the extra costs required to complete clinical research, and the diversity of available plant-based expression systems meaning that no single platform technology could be developed for industrial applications (Fischer et al. 2012). A timeline of major events in the history of molecular farming is shown in Fig. 3.1.

The biopharmaceutical industry has traditionally centered on a small number of standardized platforms. Focusing on the platform rather than the product is advantageous because the performance of each platform can be improved incrementally, the upfront investment in infrastructure can be recovered, and regulatory guidelines can evolve based on established practices, albeit at the expense of abandoning "difficult" products that are not suitable for production using accepted platform technologies. Molecular farming was a novel approach because the use of

biologically diverse systems based on plants allowed researchers to focus on the best way to make the product rather than adapting the product to the platform. However, the fragmented nature of the technology made it unattractive to industry because there was no single driving force to establish molecular farming as a competitive platform. Many of the original molecular farming SMEs collapsed, and the large industry players gradually lost interest. The more recent revival of molecular farming reflects two major advances—first, the focus on specific platforms, which paved the way for specific regulatory guidelines, and second, the focus on niche products that can be produced more successfully in plants than by bacteria or microbes (Paul et al. 2013).

3.1.2 The Development of Non-pharmaceutical Products

The pioneers of molecular farming developed plants that could be used as an upstream production system, but little attention was paid to downstream processing (DSP) or the economics of an entire manufacturing process. The first studies to consider molecular farming as a commercial process were carried out by researchers at ProdiGene Inc. (College Station, TX) and focused on the use of maize kernels to produce technical reagents and industrial enzymes (Hood et al. 1997, Kusnadi et al. 1998, Witcher et al. 1998).

The lead product was the egg protein avidin, which was already produced commercially from hens' eggs. The two most important considerations from an economic perspective were the yield (i.e., the amount of avidin produced per unit of processed plant biomass) and the stability of the product under normal processing conditions. Indeed, ProdiGene Inc. was the first to consider the DSP costs of molecular farming. The maize-derived avidin was produced with a yield of 230 mg per kg of transgenic seed, and structurally it was nearly identical to its native counterpart. Importantly, it was stable under normal maize processing conditions (storage at 10 °C, followed by dry milling, fractionation, and hexane extraction). This made it competitive with egg-derived avidin (Hood et al. 1997). The commercial success of avidin was followed by β-glucuronidase, which was produced with a yield of approximately 80 mg per kg of dry seeds. This product was also similar in structure to its native counterpart and stable under typical maize processing conditions (Witcher et al. 1998). These and several other products have been marketed by Sigma-Aldrich Fine Chemicals (St Louis, MI) as technical reagents for laboratory use.

The commercial production of recombinant proteins in maize established that molecular farming was competitive even if the product was already available from an abundant and inexpensive source and provided economic data indicating that DSP costs account for the majority of overall production costs. This is because the most expensive processing steps are generally product specific, based on the chromatography media required to isolate particular proteins. Therefore, the economic benefits of inexpensive upstream production and early processing steps are

typically overshadowed by the expensive polishing steps required regardless of the platform (Hood et al. 2002). ProdiGene Inc. can be credited for establishing the principles of DSP that were subsequently developed and applied to other crops, focusing on the removal of plant-specific contaminants such as cellulose fibers and oils (Menkhaus et al. 2004, Nikolov and Woodard 2004, Wilken and Nikolov 2012). Plants also produce certain secondary products that interfere with processing, and unique steps are needed to remove them. For example, the extraction of recombinant aprotinin from maize seeds was initially hampered by the presence of an endogenous trypsin inhibitor, which was eventually removed by introducing acid extraction and heat-denaturing steps (Azzoni et al. 2002, 2005, Zhong et al. 2007). This example also highlights the advantages of a broad range of alternative hosts in molecular farming—the same protein can be produced in tobacco without the need for additional processing steps because the troublesome protease is not present in this species (Pogue et al. 2010).

Commercial molecular farming based on seeds has also been established by other companies, which have developed economically viable DSP strategies. For example, Ventria Bioscience (Fort Collins, CO) produces several vaccines and proteins such as human albumin, transferrin, lactoferrin, and lysozyme in rice seeds. The purification of recombinant lysozyme is inhibited by the presence of phytic acid in the seed because this has a strong negative charge and competes with the cationic exchange resin used for polishing. Downstream processing therefore involves unique steps to remove the phytic acid, such as acid hydrolysis and precipitation (Wilken and Nikolov 2006, 2010). Other companies have established economic models for molecular farming involving non-pharmaceutical products, e.g., ORF Genetics (Iceland) produces human growth hormone and cytokines in barley seeds approved for diagnostic use, academic and private research, and in the case of growth hormone as a cosmetic additive (distributed by Sif Cosmetics, Iceland, and marketed in airline magazines and duty free shops).

3.2 Barriers to Pharmaceutical Molecular Farming

3.2.1 Economic Barriers

Although Prodigene Inc. found commercial success with non-pharmaceutical proteins, they also developed a number of maize lines expressing vaccine antigens and antibodies ultimately intended for human use (Streatfield et al. 2003, Hood et al. 2002, Lamphear et al. 2002). One of the problematical issues that emerged from these studies was that the higher costs of pharmaceutical development compared to industrial enzymes made molecular farming economically unfavorable unless the costs of production could be reduced.

One way to reduce production costs is to increase the yield of recombinant protein per unit of plant biomass, and it is fair to say that low yields were a major

technical hurdle faced by the pioneers of molecular farming. In the context of developing a commercially viable process, the intrinsic yield is not as important as the overall economy of the process (i.e., if the low yield per unit of plant biomass can be offset by greater scalability and larger-scale production, then it is still more economical overall than a fermenter), but this becomes increasingly untenable when DSP costs are considered. Whereas upstream production benefits from the economy of scale (i.e., it does not cost significantly more to double the number of plants producing a given protein, whereas doubling the capacity of a fermenter facility can be prohibitively expensive), the costs of downstream processing are more linear (i.e., more biomass equals more contaminants to remove equals more filter cassettes and chromatography resins, making the process overall much less economical). Therefore, despite the advantages of scalability, there is still a huge impetus to increase the intrinsic yields of recombinant proteins in plants and increase the concentration of recombinant protein in the biomass.

There are many strategies now available to increase the yields of recombinant proteins in plants, including the optimization of expression cassettes, use of expression strategies that reduce the risk of epigenetic silencing, targeting the recombinant protein to subcellular compartments that offer the greatest stability, and ensuring that plants/plant cells are maintained in the optimal environment for recombinant protein accumulation, as well as fusion protein strategies to increase protein stability (Twyman et al. 2013). Epigenetic silencing tends to affect integrated transgenes more than episomal ones, so the low yields that tend to occur in transgenic plants can often be increased by transient expression using systems based on *Agrobacterium tumefaciens* and/or engineered plant viruses, which also offer a shorter production cycle and thus more production campaigns per year (Giritch et al. 2006; Sainsbury and Lomonossoff 2008; Vézina et al. 2009; Huang et al. 2010; Pogue et al. 2010). Epigenetic effects can also be avoided by introducing DNA into the plastid genome (Cardi et al. 2010). Both transient expression and plastid transformation not only reduce epigenetic effects but also increase the active transgene copy number, thus boosting the rate of transcription. Overall, however, transgenic plants remain the most suitable platform for products required in large volumes because there is theoretically no limit to their scalability once a stable line has been established; regulatory approval is also easier for stable transgenic plants because they are recognized under EMA guidelines (see below), and only one organism needs to be regulated (Fischer et al. 2012).

There have been several recent developments to improve the efficiency of DSP for the production of pharmaceutical products from plants. Although based on the principles described above for non-pharmaceutical products, the need for quality and consistency in the manufacturing process has driven researchers to identify novel approaches to remove plant-specific contaminants and to develop models to facilitate purification. For example, flocculation (Buyel and Fischer 2014a) and heat precipitation (Buyel et al. 2014) have been shown to increase the efficiency of depth filtration during the purification of antibodies produced in tobacco leaves, and the filter train can also be optimized to remove plant-derived particulates more effectively (Buyel and Fischer 2014b). The behavior of the target protein and host cell

proteins can be modeled to improve the overall efficiency of purification, including the use of quantitative structure–activity relationship (QSAR) models to predict how host cell proteins behave during chromatography (Buyel et al. 2013b).

3.2.2 Regulatory Barriers

In addition to the historical low yields, the progress of molecular farming towards the clinic was delayed by the lack of a concrete regulatory pathway, including relevant guidelines for good manufacturing practice (GMP) relevant to plants (Spok et al. 2008, Fischer et al. 2012). Early legislation jointly drafted by the US Department of Agriculture (USDA) and the Food and Drug Administration (FDA) was flexible in terms of the platforms covered and the interpretation of GMP guidelines (FDA/USDA 2002). However, the European Medicines Agency (formerly EMEA, now EMA) based its recommendations on existing guidelines for biopharmaceutical production in mammalian cells, including concepts that are practically incompatible with plants such as cell banking and batch-to-batch consistency based on clonal identity (EMEA 2002).

These unharmonious regulations caused a schism in the molecular farming community, with some stakeholders pursuing the development of platforms based on plant cells (which were thought more compatible with the EMEA regulations) and others pushing for new guidelines suitable for whole plants (along the lines of the FDA/USDA regulations). The CONCERT™ platform based on tobacco BY-2 cells is one example of the former approach, and this was used by Dow AgroSciences to produce a poultry vaccine which was approved by the USDA in 2006 (Schillberg et al. 2013). The Israeli company Protalix BioTherapeutics followed the same route to develop the ProCellEx platform based on carrot cells, which is thus far the only plant-based platform used to manufacture biopharmaceuticals approved for human use (Aviezer et al. 2009a, b; Paul et al. 2013). Other biopharmaceutical products manufactured using plant cell/tissue platforms are also close to approval (Tiwari et al. 2009, Xu et al. 2011, Schillberg et al. 2013).

Although plant cells are conceptually similar to mammalian cells and fit better under the established regulatory guidelines, they also suffer some of the same limitations such as the dependence on fermenter infrastructure. In contrast, whole plants are much more scalable because they can be grown in greenhouses (Fig. 3.2) or other contained facilities and are thus suitable for high-volume production. The current regulatory guidelines for whole plants differ substantially in the EU and USA. The original FDA guidelines were flexible enough to incorporate any system based on whole plants or plant organs (FDA/USDA 2002), whereas even the updated EMA guidelines, published in 2008 after lengthy consultation and enacted in 2009, only consider transgenic plants and thus fail to include transient expression systems (EMEA 2009). GMP manufacturing based on transient expression in leafy crops such as tobacco and alfalfa is therefore flourishing in the USA but is still in regulatory limbo in the EU (Tremblay et al. 2010, Whaley et al. 2011). The most

Fig. 3.2 Cultivation of tobacco plants under controlled conditions. The degree of containment for plant growth can be increased, compared to common greenhouse settings, by using hydroponic systems (*left panel*) including defined substrates such as rock wool and fertilizer solutions. If a completely contained environment is preferred, phytotrons (*right panel*) can be used instead because they provide full control over temperature, atmosphere, humidity, and light

advanced plant-derived pharmaceutical products made in the EU are those produced in transgenic plants. One pioneering product was the HIV-neutralizing antibody 2G12 produced in transgenic tobacco as part of the publicly funded project Pharma-Planta, which included the development of a complete set of GMP guidelines in concert with the regulators allowing the completion of a successful phase I clinical trial (Fischer et al. 2012).

3.3 The Breakthrough into Niche Markets

One major reason for the failure of the initial pharmaceutical molecular farming "gold rush" was the attempt to displace established platforms such as CHO cells from their hegemonic position. Most disruptive technologies do not succeed by toppling established technologies in one fell swoop. Rather, they initially demonstrate their prowess in niche markets and then expand their market share. In its second commercial incarnation, pharmaceutical molecular farming is following this prescribed route and has become established in certain niche markets which most effectively demonstrate its strengths compared to traditional platforms. We consider four of these niche markets in turn.

3.3.1 Unique Properties of Plants: Edible Organs

The edible nature of many plant organs (particularly leaves, seeds, and fruits) was one of the early aspects explored by researchers. Indeed, several vaccine antigens produced in potato tubers, maize and rice seeds, lettuce leaves, and tomato fruits entered clinical trials before the new GMP directives came into force and demanded that all pharmaceutical products were produced according to GMP standards even for phase I clinical trials.

The administration of oral vaccines induces an immune response via gut-associated lymphoid tissues, which has been effective in challenge studies with many pathogens (Yusibov et al. 2011) including tandem epitopes against multiple pathogens on the same polypeptide (Soria-Guerra et al. 2011). Seeds are particularly valuable in this context because not only are they edible, they are also difficult to digest, which means that vaccine antigens expressed in seeds are released into the gut slowly thus increasing their ability to interact with immune effector cells (Hofbauer and Stoger 2013). Oral vaccines can elicit both systemic and mucosal immune responses if the antigen is protected by the plant cell matrix, and although this can also be achieved using coated formulations that dissolve slowly, the same effect can be accomplished by expressing antigens in seeds and targeting them for accumulation in protein storage compartments, particularly protein bodies in rice which are highly resistant to digestion (Ogawa et al. 1987). Prophylactic oral antibodies can also be delivered in seeds, as shown by the expression in peas of sporozoite-neutralizing anti-*Eimeria* spp. single-chain antibodies, which can be used to prevent coccidiosis in chickens (Zimmermann et al. 2009). The delivery of recombinant pharmaceutical proteins in edible plant organs also reduces or even eliminates the costs of DSP and provides specific advantages in developing countries because recombinant proteins appear to remain stable in desiccated seeds for years, even at ambient temperatures, thus removing the need for a cold chain (Stoger et al. 2005).

3.3.2 Unique Properties of Plants: Glycan Structures

The mechanism of *N*-glycosylation in plants is similar to that in humans until the nascent *N*-glycan reaches the Golgi apparatus, whereupon it is decorated with species-dependent oligosaccharide structures including β1,2-xylose and core α1,3-fucose residues (Gomord et al. 2010). In mammals, the same *N*-glycans are decorated with β1,4-galactose and sialic acid residues that are not found in plants. Glycosylation is relevant to molecular farming because glycan structures can affect the stability, targeting, immunogenicity, pharmacokinetic properties, and biological activity of a protein. The presence of plant glycans on recombinant pharmaceutical proteins has yet to show immunogenic or harmful activity in humans, but there has been a significant effort to either prevent the addition of plant glycans or humanize

the glycosylation pathway, e.g., by retrieving proteins to the endoplasmic reticulum before they are exposed to Golgi-resident enzymes or genetically modifying plants to eliminate plant-type glycosylases and introduce human ones (Sriraman et al. 2004, Triguero et al. 2005, Strasser et al. 2009, Castilho et al. 2010). This extensive research has yielded an unexpected collateral benefit, in that several groups have carried out extensive comparative tests of different protein glycoforms and have shown that the glycan profiles generated in plants can actually improve the performance of their products compared to "authentic" glycoforms produced in mammalian cells.

The primary example of a "biobetter" product containing plant glycans is Elelyso (generic name taliglucerase alfa), the only plant-derived recombinant pharmaceutical protein currently approved for use in humans. Elelyso is a recombinant form of the human enzyme glucocerebrosidase and is indicated for the treatment of the inherited disorder Gaucher's disease. It is produced by Protalix BioTherapeutics (Israel) using the ProCellEx platform based on carrot cells, and it accumulates in the vacuole. This is important because vacuolar targeting preserves the terminal mannose residues on the glycan chains, which are essential for efficient uptake by macrophages in humans and thus required for therapeutic efficacy (Shaaltiel et al. 2007). In contrast, the equivalent product manufactured in CHO cells (Cerezyme (imiglucerase) produced by Genzyme Corp.) has terminal sialic acid residues that must be enzymatically removed in vitro to expose the critical mannose residues. The plant-derived product is thus advantageous because downstream processing is more cost-effective. In other cases, the biological activity of the product improves due to the presence of plant glycans. For example, Synthon BV (which recently purchased LEX technology based on the aquatic plant *Lemna minor* from Biolex Therapeutics) produces "glyco-optimized" proteins that have distinct glycan profiles and thus better pharmacokinetic properties compared to their counterparts derived from mammalian cells. The lead product is a therapeutic CD30-specific antibody indicated for non-Hodgkin's lymphoma, which has a tenfold higher affinity for its receptor and 20-fold greater antibody-dependent cellular cytotoxicity (ADCC) against tumor cells (Cox et al. 2006).

3.3.3 The Promise of Rapid Scalability

The advantage of rapid recombinant protein production in concert with the scalability of crops such as tobacco (Fig. 3.2) and alfalfa provides a niche market for transient expression in plants (D'Aoust et al. 2008, 2010). The most effective way to respond to an emerging pandemic or bioterrorist threat is to deploy strategic vaccines, but traditional production methods take a long time to scale up and would not be able to cope with a rapidly spreading contagious disease such as influenza. Models suggest that it would take up to 6 months to reach production targets for a pandemic influenza vaccine in western countries using egg-based production, but that the disease would peak within 3 months (Rappuoli and Dormitzer 2012).

However, several organizations now offer GMP manufacturing based on transient expression in tobacco, or its close relative *Nicotiana benthamiana*, including Kentucky BioProcessing (Owensboro, KY), Icon Genetics (Halle, Germany), Fraunhofer CMB (Newark, DE), and Medicago (Quebec, Canada). An additional facility is being constructed by Texas A&M University (College Station, TX) and G-Con, LCC.

Transient expression systems produce large amounts of recombinant protein rapidly (milligram quantities per plant within a few days) and can be scaled up quickly, currently providing the only reliable platform for rapid-response situations. Medicago Inc. found that their alfalfa-based transient expression system could provide batches of viruslike particles for vaccination against H1N1 and H5N1 strains of influenza within 3 weeks of receiving the hemagglutinin and neuraminidase gene sequences (Pandey et al. 2010, Landry et al. 2010). As an indication that the pharmaceutical industry is once again taking an active interest in molecular farming, the Japanese pharmaceutical company Mitsubishi Tanabe Pharma Corp. acquired a majority shareholding of Medicago Inc. in 2013, with most of the remaining shares owned by Philip Morris Investments. The Fraunhofer Center for Molecular Biotechnology (Newark, DE) has also produced GMP-compliant vaccines in a few weeks from receipt of sequence by transient expression in tobacco using their iBioLaunch platform, including influenza hemagglutinin subunits from strains H3N2, H5N1, and H1N1 at yields between 50 and 200 mg of recombinant protein per kg of fresh leaves (Shoji et al. 2008, 2011).

3.3.4 The Promise of Agricultural Production Scales

Whereas some recombinant proteins are required in large amounts rapidly, others are required in immense amounts continuously, and plants again provide a solution that cannot be met by other production platforms. An example of such a product is 2G12, the HIV-neutralizing antibody described above, which can be used as part of a microbicidal cocktail to prevent HIV infections when applied before sexual intercourse (Ramessar et al. 2010). Effective protection requires the repetitive application of milligram quantities of antibody. This would be prohibitively expensive if produced using conventional fermenter technology, and the demand for such an antibody would rapidly outstrip the entire global capacity for GMP production if the product were to be sold as a conventional pharmaceutical product in the developed world. However, the target population is predominantly in sub-Saharan Africa and includes the world's poorest people. The only way to meet this demand is to grow pharmaceutical crops on a vast scale using local infrastructure, a concept developed in the Pharma-Planta project (Fischer et al. 2012). The driving force in this scenario is scale not speed, and the best platform is transgenic plants, which can be grown on a massive scale even in contained greenhouses without skilled labor allowing the production and processing of 200–1,000 kg batches of antibodies at a fraction of the cost of fermenters. Functional HIV-neutralizing antibodies have

been produced in tobacco for clinical trials (Fig. 3.2) and also in maize seeds as a competitive platform for the development of novel microbicides intended for deployment in developing countries (Ramessar et al. 2008, Rademacher et al. 2008). Fraunhofer IME and the Pharma-Planta consortium were instrumental in establishing the EU guidelines and regulatory framework for the manufacture of clinical-grade monoclonal antibodies in transgenic tobacco plants. Fraunhofer IME has scaled its productivity and downstream processing to up to 500 kg in a single day and will start operating a fully automated vertical farming unit by mid-2014.

3.4 The Future of Molecular Farming

Molecular farming in plants has many technical and economic advantages that make it commercially attractive, but the biopharmaceutical industry has become reliant on a small number of standardized and approved platform technologies and has invested heavily in the corresponding infrastructure. The established technologies have evolved by incremental improvements and can produce yields of recombinant protein at least an order of magnitude higher than any plant-based system. Therefore, rather than seeking to displace these incumbent technologies by direct competition, molecular farming is now evolving as a disruptive technology, providing game-changing benefits in a small number of niche markets. These benefits include optimized glycans, versatility to adapt to market forces that cannot be satisfied with the current platforms (individualized therapies, rapid-response vaccines, bulk "commodity" antibody manufacturing), and unique properties of cereal seeds that provide an efficient strategy for oral vaccination. As further technical developments unfold, the number of market needs that can be met by plants will increase. For example, the combination of rapid response and bioencapsulation for oral vaccines could be met by the production of vaccine antigens in tobacco plastids, as recently shown for the production of coagulation factor IX in tobacco leaves and the subsequent demonstration that orally administered tobacco leaves can prevent anaphylactic reactions in a mouse model of hemophilia B (Verma et al. 2010). The value of incorporating quality by design (QbD) principles based on the early implementation of design of experiments (DOE) in process development has recently been shown to increase yields and batch-to-batch consistency in both transgenic and transient systems, which will help to make molecular farming even more competitive (Buyel and Fischer 2012; Buyel et al. 2013a). The move towards increased quality in the production process is in line with the latest ICH guidelines, which recommend the inclusion of inline process analytical technology (PAT) to ensure consistency, as well as rigorous quality assurance and quality control (QA/QC) during the manufacturing process (ICH 2012). The innovation demonstrated within the field of molecular farming, and the resulting versatility and adaptability, will continue to be the driving force that ensures the commercial success of this exciting and promising technology if more product candidates can be brought to the market.

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References

Aviezer D, Almon-Brill E, Shaaltiel Y et al (2009a) Novel enzyme replacement therapy for Gaucher disease: ongoing Phase III clinical trial with recombinant human glucocerebrosidase expressed in plant cells. Mol Genet Metab 96:S13–S14

Aviezer D, Brill-Almon E, Shaaltiel Y et al (2009b) A plant-derived recombinant human glucocerebrosidase enzyme—a preclinical and phase I investigation. PLoS One 4:e4792

Azzoni AR, Kusnadi AR, Miranda EA et al (2002) Recombinant aprotinin produced in transgenic corn seed: extraction and purification studies. Biotechnol Bioeng 80:268–276

Azzoni AR, Farinas CS, Miranda EA (2005) Transgenic corn seed for recombinant protein production: relevant aspects on the aqueous extraction of native components. J Sci Food Agric 85:609–614

Bevan MW, Flavell RB, Chilton MD (1983) A chimaeric antibiotic resistance gene as a selectable marker for plant cell transformation. Nature 304:184–187

Buyel JF, Fischer R (2012) Predictive models for transient protein expression in tobacco (*Nicotiana tabacum* L.) can optimize process time, yield, and downstream costs. Biotechnol Bioeng 109:2575–2588

Buyel JF, Fischer R (2014a) Flocculation increases the efficacy of depth filtration during the downstream processing of recombinant pharmaceutical proteins produced in tobacco. Plant Biotechnol J 12:240–252

Buyel JF, Fischer R (2014b) Scale-down models to optimize a filter train for the downstream purification of recombinant pharmaceutical proteins produced in tobacco leaves. Biotechnol J 9:415–425

Buyel JF, Kaever T, Buyel JJ et al (2013a) Predictive models for the accumulation of a fluorescent marker protein in tobacco leaves according to the promoter/5'UTR combination. Biotechnol Bioeng 110:471–482

Buyel JF, Woo JA, Cramer SM et al (2013b) The use of quantitative structure–activity relationship models to develop optimized processes for the removal of tobacco host cell proteins during biopharmaceutical production. J Chromatogr A 1322:18–28

Buyel JF, Gruchow HM, Boes A et al (2014) Rational design of a host cell protein heat precipitation step can simplify the subsequent purification of recombinant proteins from tobacco. Biotechnol Bioeng 88:162–170

Cardi T, Lenzi P, Maliga P (2010) Chloroplasts as expression platforms for plant-produced vaccines. Expert Rev Vaccines 9:893–911

Castilho A, Strasser R, Stadlmann J et al (2010) In planta protein sialylation through overexpression of the respective mammalian pathway. J Biol Chem 285:15923–15930

Cox KM, Sterling JD, Regan JT et al (2006) Glycan optimization of a human monoclonal antibody in the aquatic plant *Lemna minor*. Nat Biotechnol 24:1591–1597

D'Aoust MA, Couture MM, Charland N et al (2010) The production of hemagglutinin-based virus-like particles in plants: a rapid, efficient and safe response to pandemic influenza. Plant Biotechnol J 8:607–619

D'Aoust MA, Lavoie PO, Couture MM et al (2008) Influenza virus-like particles produced by transient expression in *Nicotiana benthamiana* induce a protective immune response against a lethal viral challenge in mice. Plant Biotechnol J 6:930–940

EMEA (2002) Committee for Proprietary Medicinal Products (CPMP). Points to consider on quality aspects of medicinal products containing active substances produced by stable transgene expression in higher plants (EMEA/CPMP/BWP/764/02). EMA, London, UK
EMEA (2009) Committee for Proprietary Medicinal Products (CPMP). Guideline on the quality of biological active substances produced by stable transgene expression in higher plants (EMEA/CHMP/BWP/48316/2006). EMA, London, UK
FDA/USDA (2002) Draft guidance. Drugs, biologics, and medical devices derived from bioengineered plants for use in humans and animals. FDA, Rockville, MD. http://www.fda.gov/downloads/AnimalVeterinary/GuidanceComplianceEnforcement/GuidanceforIndustry/UCM055424.pdf
Fischer R, Schillberg S, Hellwig S et al (2012) GMP issues for plant-derived recombinant proteins. Biotechnol Adv 30:434–439
Giritch A, Marillonnet S, Engler C et al (2006) Rapid high-yield expression of full-size IgG antibodies in plants coinfected with noncompeting viral vectors. Proc Natl Acad Sci USA 103:14701–14706
Gomord V, Fitchette AC, Menu-Bouaouiche L et al (2010) Plant-specific glycosylation patterns in the context of therapeutic protein production. Plant Biotechnol J 8:564–587
Hiatt AH, Caffertey R, Bowdish K (1989) Production of antibodies in transgenic plants. Nature 342:76–78
Hofbauer A, Stoger E (2013) Subcellular accumulation and modification of pharmaceutical proteins in different plant tissues. Curr Pharm Des 19:5495–5502
Hood EE (2002) From green plants to industrial enzymes. Enzyme Microb Technol 30:279–283
Hood EE, Witcher DR, Maddock S et al (1997) Commercial production of avidin from transgenic maize: characterization of transformant, production, processing, extraction and purification. Mol Breed 3:291–306
Hood EE, Woodard SL, Horn ME (2002) Monoclonal antibody manufacturing in transgenic plants—myths and realities. Curr Opin Biotechnol 13:630–635
Huang Z, Phoolcharoen W, Lai H et al (2010) High-level rapid production of full-size monoclonal antibodies in plants by a single-vector DNA replicon system. Biotechnol Bioeng 106:9–17
International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) (2012) Q11. Development and manufacture of drug substances (chemical entities and biotechnological/biological entities). Fed Regist 77:69634–69635
Kusnadi AR, Evangelista RL, Hood EE et al (1998) Processing of transgenic corn seed and its effect on the recovery of recombinant β-glucuronidase. Biotechnol Bioeng 60:44–52
Lamphear BJ, Streatfield SJ, Jilka JM et al (2002) Delivery of subunit vaccines in maize seed. J Control Release 85:169–180
Landry N, Ward BJ, Trepanier S et al (2010) Preclinical and clinical development of plant-made virus-like particle vaccine against avian H5N1 influenza. PLoS One 5:e15559
Ma JKC, Drake PMW, Christou P (2003) The production of recombinant pharmaceutical proteins in plants. Nat Rev Genet 4:794–805
Menkhaus TJ, Bai Y, Zhang CM et al (2004) Considerations for the recovery of recombinant proteins from plants. Biotechnol Prog 20:1001–1014
Nikolov ZL, Woodard SL (2004) Downstream processing of recombinant proteins from transgenic feedstock. Curr Opin Biotechnol 15:479–486
Ogawa M, Kumamaru T, Satoh H et al (1987) Purification of protein body-I of rice seed and its polypeptide composition. Plant Cell Physiol 28:1517–1527
Pandey A, Singh N, Mittal SK (2010) Egg-independent vaccine strategies for highly pathogenic H5N1 influenza viruses. Hum Vaccin 6:178–188
Paul MJ, Teh AY, Twyman RM et al (2013) Target product selection—where can molecular pharming make the difference? Curr Pharm Des 19:5478–5485

Pogue GP, Vojdani F, Palmer KE et al (2010) Production of pharmaceutical-grade recombinant aprotinin and a monoclonal antibody product using plant-based transient expression systems. Plant Biotechnol J 8:638–654
Rademacher T, Sack M, Arcalis E et al (2008) Recombinant antibody 2G12 produced in maize endosperm efficiently neutralizes HIV-1 and contains predominantly single-GlcNAc N-glycans. Plant Biotechnol J 6:189–201
Ramessar K, Rademacher T, Sack M et al (2008) Cost-effective production of a vaginal protein microbicide to prevent HIV transmission. Proc Natl Acad Sci USA 105:3727–3732
Ramessar K, Sabalza M, Miralpeix B et al (2010) Can microbicides turn the tide against HIV? Curr Pharm Des 16:468–485
Rappuoli R, Dormitzer PR (2012) Influenza: options to improve pandemic preparation. Science 336:1531–1533
Sainsbury F, Lomonossoff GP (2008) Extremely high-level and rapid protein production in plants without the use of viral replication. Plant Physiol 148:1212–1218
Schillberg S, Raven N, Fischer R et al (2013) Molecular farming of pharmaceutical proteins using plant suspension cell and tissue cultures. Curr Pharm Des 19:5531–5542
Shaaltiel Y, Bartfeld D, Hashmueli S et al (2007) Production of glucocerebrosidase with terminal mannose glycans for enzyme replacement therapy of Gaucher's disease using a plant cell system. Plant Biotechnol J 5:579–590
Shoji Y, Chichester JA, Bi H et al (2008) Plant-expressed HA as a seasonal influenza vaccine candidate. Vaccine 26:2930–2934
Shoji Y, Chichester JA, Jones M et al (2011) Plant-based rapid production of recombinant subunit hemagglutinin vaccines targeting H1N1 and H5N1 influenza. Hum Vaccin 7:41–50
Soria-Guerra RE, Rosales-Mendoza S, Moreno-Fierros L et al (2011) Oral immunogenicity of tomato-derived sDPT polypeptide containing *Corynebacterium diphtheriae, Bordetella pertussis* and *Clostridium tetani* exotoxin epitopes. Plant Cell Rep 30:417–424
Spok A, Twyman RM, Fischer R et al (2008) Evolution of a regulatory framework for plant-made pharmaceuticals. Trends Biotechnol 26:506–517
Sriraman R, Bardor M, Sack M et al (2004) Recombinant anti-hCG antibodies retained in the endoplasmic reticulum of transformed plants lack core-xylose and core-α(1,3)-fucose residues. Plant Biotechnol J 2:279–287
Stoger E, Sack M, Nicholson L et al (2005) Recent progress in plantibody technology. Curr Pharm Des 11:2439–2457
Strasser R, Castilho A, Stadlmann J et al (2009) Improved virus neutralization by plant-produced anti-HIV antibodies with a homogeneous β1,4-galactosylated N-glycan profile. J Biol Chem 284:20479–20485
Streatfield SJ, Lane JR, Brooks CA et al (2003) Corn as a production system for human and animal vaccines. Vaccine 21:812–815
Tiwari S, Verma PC, Singh PK et al (2009) Plants as bioreactors for the production of vaccine antigens. Biotechnol Adv 27:449–467
Tremblay R, Wang D, Jevnikar AM et al (2010) Tobacco, a highly efficient green bioreactor for production of therapeutic proteins. Biotechnol Adv 28:214–221
Triguero A, Cabrera G, Cremata JA et al (2005) Plant-derived mouse IgG monoclonal antibody fused to KDEL endoplasmic reticulum-retention signal is N-glycosylated homogeneously throughout the plant with mostly high-mannose-type N-glycans. Plant Biotechnol J 3:449–457
Twyman RM, Stoger E, Schillberg S et al (2003) Molecular farming in plants: host systems and expression technology. Trends Biotechnol 21:570–578
Twyman RM, Schillberg S, Fischer R (2005) Transgenic plants in the biopharmaceutical market. Expert Opin Emerg Drugs 10:185–218
Twyman RM, Schillberg S, Fischer R (2013) Optimizing the yield of recombinant pharmaceutical proteins in plants. Curr Pharm Des 19:5486–5494

Verma D, Moghimi B, LoDuca PA et al (2010) Oral delivery of bioencapsulated coagulation factor IX prevents inhibitor formation and fatal anaphylaxis in hemophilia B mice. Proc Natl Acad Sci USA 107:7101–7106

Vézina LP, Faye L, Lerouge P et al (2009) Transient co-expression for fast and high-yield production of antibodies with human-like N-glycans in plants. Plant Biotechnol J 7:442–455

Whaley KJ, Hiatt A, Zeitlin L (2011) Emerging antibody products and Nicotiana manufacturing. Hum Vaccin 7:349–356

Wilken LR, Nikolov ZL (2006) Factors influencing recombinant human lysozyme extraction and cation exchange adsorption. Biotechnol Prog 22:745–752

Wilken LR, Nikolov ZL (2010) Evaluation of alternatives for human lysozyme purification from transgenic rice: impact of phytic acid and buffer. Biotechnol Prog 26:1303–1311

Wilken LR, Nikolov ZL (2012) Recovery and purification of plant-made recombinant proteins. Biotechnol Adv 30:419–433

Witcher D, Hood EE, Peterson D et al (1998) Commercial production of β-glucuronidase (GUS): a model system for the production of proteins in plants. Mol Breed 4:301–312

Xu J, Ge X, Dolan MC (2011) Towards high-yield production of pharmaceutical proteins with plant cell suspension cultures. Biotechnol Adv 29:278–299

Yusibov V, Streatfield SJ, Kushnir N (2011) Clinical development of plant-produced recombinant pharmaceuticals. Vaccines, antibodies and beyond. Hum Vaccin 7:313–321

Zhong Q, Xu L, Zhang C et al (2007) Purification of recombinant aprotinin from transgenic corn germ fraction using ion exchange and hydrophobic interaction chromatography. Appl Microbiol Biotechnol 76:607–613

Zimmermann J, Saalbach I, Jahn D et al (2009) Antibody expressing pea seeds as fodder for prevention of gastrointestinal parasitic infections in chickens. BMC Biotechnol 9:79

Chapter 4
TrypZean™: An Animal-Free Alternative to Bovine Trypsin

Aparna Krishnan and Susan L. Woodard

4.1 Introduction

4.1.1 What Is Trypsin?

Bovine trypsin is a well-studied and widely used enzyme in the biological sciences. It is expressed in zymogen form in mammalian pancreatic tissue along with other digestive enzymes. It is activated in the duodenum by enterokinase, which is specific for the Asp-Asp-Asp-Asp-Lys sequence that is part of the Val-Asp-Asp-Asp-Asp-Lys hexapeptide that distinguishes trypsin from trypsinogen. Once enterokinase cleaves this hexapeptide, active trypsin can autocatalytically activate more trypsinogen to active form since it has a preference for cleaving after basic amino acids such as lysine (Lys) and arginine (Arg) (Stryer 1995).

Trypsin is a member of the chymotrypsin family (Rawlings and Barrett 1994) and shares many properties with other proteolytic enzymes such as chymotrypsin and elastase. One feature of this family of serine proteases is the catalytic triad (also referred to as a charge-relay network) involving a nucleophilic serine, a histidine, and an aspartic acid residue in the binding pocket of the enzyme (Stryer 1995). Huber and Bode (1978) described trypsin as having a secondary structure dominated by two β-barrels whose interior is largely comprised of hydrophobic amino acids. Water molecules fill hollows in the structure and are integral to both the trypsinogen and trypsin forms.

The earliest studied trypsins were those obtained from bovine pancreas. β-Trypsin is the single-chain form that results when the activation peptide is

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removed from trypsinogen (Davie and Neurath 1955). Additional cleavages can occur resulting in a two-chain form called α-trypsin (Schroeder and Shaw 1968) and a further cleaved form called pseudotrypsin (Keil 1971). These multichain forms are held together by trypsin's six disulfide bonds so they are difficult to distinguish on the basis of activity (Keil 1971).

4.1.2 Use of Trypsin in Biopharmaceutical Manufacturing

Trypsin obtained either from bovine or porcine pancreas is widely used in biopharmaceutical manufacturing. There are three main ways that it is used: (1) in conjunction with EDTA to facilitate the detachment of adherent cell lines from tissue culture flasks so that the cells can be passaged; (2) in the processing of proteins, for example, the processing of proinsulin to insulin using trypsin and carboxypeptidase (Kemmler et al. 1971); and (3) in vaccine production where it is used to promote viral infectivity, for example, in influenza vaccine production where trypsin is needed by most strains to gain entry to the host cell and replicate.

There are numerous other applications for trypsin, but none of them are in widespread use. One of the minor applications worth mentioning is its use in treatment of bovine embryos to inactivate bovine viruses in tissues used during the in vitro fertilization process. Another area where trypsin use is growing is in the isolation and expansion of stem cells. These applications will be expanded upon later in this chapter (see Sect. 4.5).

4.1.3 Need for an Alternative to Animal Source Trypsin

There are many compelling reasons to avoid the use of animal-derived trypsin in various pharmaceutical applications. Animal-derived proteins can be a source of mammalian viruses (Merten 2002). With the addition of a viral inactivation step to the purification train for most biopharmaceutical purification processes, the risk for an approved biopharmaceutical to be the source of a porcine- or bovine-derived virus should be very low. However, some viruses are particularly resistant to the typical viral inactivation protocol. In addition, some live virus vaccines cannot be put through a viral inactivation step. Contamination of two approved pediatric rotavirus vaccines with porcine viral DNA sequences was subsequently attributed to the use of trypsin in the cell banks used for the manufacture of the vaccines (McClenahan et al. 2011). Since this porcine virus is not known to be infectious to humans, the vaccines remain on the market. It is likely that the manufacturers are working on alternatives to these vaccines, but they will take a long time to redevelop on new cell material.

Another compelling reason to look for an alternative to animal-derived trypsin is the potential for contamination from other pathogens such as prions, the causative

agents of a family of transmissible spongiform encephalopathies (TSE). Prions are misfolded proteins that are thought to convert other proteins to the misfolded type by association. Although there are still many unanswered questions regarding the mechanism of this conversion, this family of diseases shares a common spongy brain degeneration pathology which ultimately results in death. The best known TSE is bovine spongiform encephalopathy also referred to as “mad cow” disease. Due to the misfolded nature of these prion proteins, they cannot be easily removed or inactivated. Detection is also difficult. This risk is difficult to assess but the fear of prion transmission through biologics is certainly a major factor in driving a move toward animal-origin free (AOF) reagents in biopharmaceutical manufacturing.

Finally, there is a possibility that contaminants such as mycoplasma could be present in trypsin preparations used in cell culture. Some bovine-sourced reagents, such as fetal calf serum, are known to harbor mycoplasma strains. However, one of the most common strains of mycoplasma found in cell cultures is *Mycoplasma hyorhinis*, a strain most commonly associated with pigs. A paper in 1990 demonstrated that this mycoplasma strain can survive storage in trypsin solutions (Polak-Vogelzang et al. 1990), so it is plausible that porcine-derived trypsin is the source of some mycoplasma contamination of cell cultures in which it was used.

Additional regulatory hurdles may now exist for those developing processes that use animal-derived trypsin. A CBER “Guidance for Industry” subtitled “Characterization and qualification of cell substrates and other biological materials used in the production of viral vaccines for infectious disease indications” (http://www.fda.gov/downloads/biologicsbloodvaccines/Guidancecomplianceregulatoryinformation/guidances/vaccines/ucm202439.pdf) (2010) has a section on trypsin indicating the extra testing that should be performed depending on whether the trypsin is of bovine or porcine origin. A newly released draft “Guideline on the use of porcine trypsin used in the manufacture of human biological medicinal products” from the European Medicines Agency (http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2013/03/WC500139532.pdf) (2013) recommends that two different cell lines be used to test for adventitious agents that could be found in porcine trypsin. This additional testing underscores the need for alternatives to these animal sources of trypsin.

4.2 An Alternative Plant-Made Bovine Trypsin

4.2.1 Expression of Bovine Trypsin in Maize

Because trypsin is autocatalytic, it is difficult to maintain trypsinogen in the zymogen form. Once active, the enzyme can be detrimental to the host since trypsin is a particularly aggressive proteolytic enzyme. This activity limits its expression in heterologous hosts. Creative strategies are therefore required to minimize trypsin activity and achieve high expression in host systems.

Table 4.1 Effect of expression strategy on recovery of events and bovine trypsin activity in seed from transgenic maize

Construct name	Form expressed	Promoter used or target tissue	Signal sequence used	No. of events recovered	High T1 seed (TSP)[a] (%)
TRC	Zymogen	Maize polyubiquitin-1	mo-BAASS[b]	15	0.057
TRD	Active	Maize polyubiquitin-1	mo-BAASS	5	0.010
TRE	Zymogen	Endosperm promoter	Amyloplastid	10	0.75
TRF	Zymogen	Globulin-1 (germ)	mo-BAASS	16	3.3

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[a]High seed based on enzyme activity out of a subset analyzed from each ear generated per event

[b]mo-BAASS stands for maize-optimized barley alpha-amylase signal sequence (Rogers 1985)

Trypsin was produced in maize by researchers at ProdiGene, initially started when they were at Pioneer Hi-Bred (now DuPont Pioneer) where they also expressed trypsin in canola (rape) seed. Their work indicated that commercially viable levels of trypsin were obtained when it was expressed in zymogen form. Building on this work, several constructs were prepared for expression in maize using trypsin in both the active and zymogen forms along with a combination of promoters and targeting sequences.

Some of the combinations of gene, promoter, and subcellular targeting sequences used in order to obtain high levels of trypsin expression in maize seed are described in Hood and Woodard (2002). The work describes the number of recovered transformation events and the expression level of trypsin in the highest seed from all the constructs after transformants were propagated and then outcrossed to make seed. In one case, active trypsin was expressed under a constitutive promoter using the barley alpha-amylase signaling sequence (BAASS) to target the enzyme to the apoplastic space, but the expression of trypsin in apoplast was clearly detrimental to the recovery of transformation events and plant health. Fewer seeds were recovered from the plants that were able to go on and make seed, and the highest level of trypsin was only 0.01 % of total soluble protein (TSP). When the same strategy was used but the gene sequence used was trypsinogen instead of trypsin, three times as many events were recovered, and the highest seed expression was 0.057 % of TSP (Table 4.1) when measured by enzyme activity.

Seed-specific promoters further improved trypsin expression when the zymogen form was used. Using an endosperm-specific promoter along with an amyloplast targeting sequence to express trypsinogen in starch bodies, the highest level of trypsin in a single seed from the plants generated from ten events was 0.75 % of TSP. When trypsinogen was targeted to the cell wall using the BAASS, but under an embryo-specific promoter, trypsin levels were as high as 3.3 % of TSP. Clearly, expressing trypsin in zymogen form in combination with the use of seed-specific promoters was key to high levels of expression. The data obtained for the maize constructs described here, together with the canola findings mentioned earlier,

formed the basis of a patent covering protease expression in transgenic plants where the protease is expressed in its zymogen form (Howard and Hood 2000).

This seed material was put into a corn breeding program with the opposing goals of both providing a large quantity of high-expressing seed quickly and backcrossing high-expressing lines into elite backgrounds over many generations, so that hybrid seed could eventually be made. By the fifth generation, 1,000,000 kg of grain was produced expressing 58 mg of trypsin/kg, enough to yield several kilograms of purified trypsin (Woodard et al. 2003).

This material could be stored as whole seed until it was needed; could be ground into whole flour; or could be fractionated to obtain the germ fraction, defatted, dried, and ground into germ flour. Ground whole flour expressing trypsin was monitored over the course of a year when stored at both room temperature and at 50 °F/50 % humidity, and no loss of trypsin activity was observed (Mayor and Woodard, unpublished). The germ flour behaved similarly but was only monitored for about 6 months. Theoretically, a large enrichment of trypsin should result upon fractionation since the embryo makes up a small portion of the total seed and trypsin was targeted to the embryo (or the germ fraction). Fractionation is not always a precise process, however, and theoretical enrichment levels are typically not realized. If a germ fraction is prepared, it will be high in oil and will need to be defatted in order to prevent the germ from going rancid. It is not clear if the endogenous protease inhibitors present in whole corn seed are localized to the germ, endosperm, or pericarp of the seed. Removing a portion of this inhibitor pool from the enriched germ fraction could be problematic if the stability of trypsinogen is affected by this fractionation.

Considering that fractionation can result in loss of trypsin-containing material, the decision to fractionate may depend more on the practicality of storage of whole grain. If half the material is lost in the process of enriching trypsin threefold, factors such as storage costs and reduced buffer extraction costs need to be weighed. By-product credits are another important consideration (Nikolov and Hammes 2002). It might be possible to use the endosperm (or starch-rich portion) in bioethanol production. These are factors that need to be considered when dealing with industrial quantities of starting material.

4.2.2 Extraction and Purification

Regardless of the expression platform, product recovery and purification generally comprise the majority cost of manufacturing a biopharmaceutical product. The relatively low cost of production for some transgenic plants means that the downstream costs could constitute an even higher proportion of the overall cost of manufacturing a product from transgenic plant platforms (Kusnadi et al. 1997). Evangelista et al. (1998) modeled production costs for purified glucuronidase from transgenic maize and found that the downstream costs were 80 % of the total cost. Therefore, designing an extraction process that maximizes the amount of

recombinant protein and minimizes the amount of host proteins is desirable because a less complex extract will potentially require fewer steps in a purification process train. For trypsinogen, the relative instability of trypsin at neutral pH led to the exploration of using very low pH to minimize autocatalysis. This approach offers an advantage in purification because, at low pH, the majority of maize seed proteins are not extracted (Woodard, unpublished results).

The small-scale purification reported by Woodard et al. (2003) was not particularly efficient. It was performed on a pool of early grain with lower trypsin levels, and the buffer-to-tissue ratio used was only 3:1. The extract, which contained only 3.5 mg of trypsin by activity assay, was treated with soybean trypsin inhibitor (SBTI) agarose beads to capture the trypsin in a batch mode by raising the pH to neutral. Once bound, trypsin activity was eluted from the SBTI agarose in a two-step process. The first step involved lowering the pH to 3.8 in order to elute a cleaved trypsin form as described in Liepnieks and Light (1974). About 20 % of the trypsin activity was present in this eluate. Next, intact trypsin was eluted by addition of a pH 2.8 buffer. Fractions of this eluate with high trypsin activity were combined and then loaded onto an SP Sepharose cation exchange chromatography column. After a salt wash at low pH, a linear gradient of both increasing salt and pH was used to elute the trypsin and obtain it in highly purified form. This small-scale purification only gave a 25 % yield of extracted trypsin. For large-scale production, a higher buffer-to-tissue ratio is used as well as more conventional process steps including ion-exchange chromatography using a salt gradient.

Optimal extraction of the enzyme from the ground corn flour, whether whole or a germ fraction, relies on a variety of parameters including the fineness of the flour, the buffer-to-tissue ratio used in the extraction, the composition of the extraction buffer, steeping time, and mixing. When working on a large scale, these factors influence process economics. Many of these parameters were examined in the early development of TrypZean™ at ProdiGene.

Large volumes of extract with suspended solids can be clarified using industrial centrifuges or a filter press to remove particulates. Further clarification may be needed in the form of a depth filter with a micron range pore size. After depth filtration, the extract can be concentrated against a low molecular weight cutoff membrane and conditioned with buffer in preparation for loading onto an SP Sepharose cation exchange column. Bound trypsin can then be eluted using salt. The eluted material can be diafiltered into the appropriate buffer for loading onto an affinity resin column. Where the lab-scale purification used an SBTI agarose column, a less expensive and more robust benzamidine Sepharose affinity resin could be substituted for larger scale work. Finally, trypsin eluted from this column could be diafiltered into a low ionic strength solution in preparation for lyophilization. The process described here should result in a high specific activity powder of greater than 95 % purity. When done on a large scale, this process is expected to have approximately 50 % yield. Although the production specifications for TrypZean™ produced in Sigma-Aldrich's animal-free manufacturing facility are proprietary, a process yield of >50 % is a common target for a commercial purification process.

4.3 Characterization and Performance

4.3.1 Characterization

Trypsin purified on the lab scale as described in Woodard et al. (2003), or on a larger scale by a process similar to the one described above (Sect. 4.3.2), had similar purity and specific activity. Trypsin activity assays can be tricky due to the fact that the enzyme specifically degrades proteins and even itself. Evidence supporting this difficulty is shown in Hood and Woodard (2002) where trypsin levels in different seed extracts were compared using three different assays. Using ELISA with antibodies to trypsinogen, measured against a standard curve of commercially available bovine trypsinogen, showed low levels ($\leq$0.1 % of TSP) in the seed extracts. When the ELISA capture time was reduced, the standard curve gave a similar result, but higher trypsinogen levels were detected (Woodard and Mayor, unpublished). This increase in level with decreased capture time could indicate that the enzyme was in its active form, degrading the capture antibody and not in the zymogen form as expected for this construct. Enzyme activity assays confirmed that trypsin was, in fact, present in active form at levels $>$1 % of TSP. Western blots were in agreement with the activity assay results (Hood and Woodard 2002).

Table 4.2 summarizes the results of various characterization assays that were performed side by side for a high purity commercially available bovine trypsin powder and bovine trypsin purified from maize. While SDS-PAGE analysis of the maize-derived trypsin showed three bands as opposed to the single band for the native protein, amino acid sequencing revealed that each band had the same N-terminal sequence as bovine trypsin (Woodard et al. 2003). Faint staining of the upper bands using a glycoprotein stain suggested presence of glycoforms in recombinant trypsin preparations. The MALDI-TOF mass spectrum of maize-derived trypsin showed a mass-to-charge ratio of approximately 23,300 Da similar to bovine trypsin but with at least six additional peaks shifted to higher mass-to-charge ratios and separated by about 200 Da, suggestive of different glycoforms. Despite the presence of multiple forms, recombinant trypsin was equivalent in terms of activity, reactivity, and protein stability to highly purified bovine trypsin.

Since characterization data indicate that the recombinant trypsin from maize was functionally equivalent to bovine-derived trypsin, it was of particular interest to know as much as possible about the nature of the one major difference, glycosylation. Native bovine trypsin is not glycosylated nor does the enzyme have any consensus sites for N-linked glycosylation. It seemed appropriate to conclude that the enzyme when purified from transgenic maize seed must have O-linked glycans since bovine trypsin has numerous potential sites for O-linked glycosylation (Woodard et al. 2003). Attempts to deglycosylate the enzyme with either an O-glycosidase or an N-glycosidase were unsuccessful.

Scientists at Sigma-Aldrich, distributors of TrypZean™, continued work on characterizing the glycosylation of this product using a variety of conventional approaches (Ray and Jalili 2011). The traditional bottom-up approach commonly

Table 4.2 Comparison of properties for maize-derived bovine trypsin to native bovine trypsin

Analysis	Information gained	Corn produced	Native trypsin	References
SDS-PAGE	Molecular weight (crude)	24, 26, and 27 kDa bands	24 kDa band	Woodard et al. (2003)
Western blot	Identity to trypsin	All bands react with Ab	Single band reacts	Woodard et al. (2003)
Mass spec	Mass-to-charge ratio	23,294	23,293 (theory)	Ray and Jalili (2011)
Glycostain	Glycosylated protein	Faint staining of upper band	No staining detected	Woodard et al. (2003)
NH_2-term sequence	Identity to trypsin	First 12 AAs sequence as trypsin	Not determined	Woodard et al. (2003)
OD 280	Purity	99 %	98 %	Woodard et al. (2003)
Specific activity[a]	Quality	3,360 USP U/mg	3,190 USP U/mg	Woodard et al. (2003)
Specific activity[b]	Quality	4,396 USP U/mg	4,268 USP U/mg	Ray and Jalili (2011)
Chymotrypsin activity	Impurity	$\leq$5 USP U/2,500 U trypsin	6.6 USP U/2,500 U trypsin	Ray and Jalili (2011)
pH optimum	Activity profile	8.6	8.7	Woodard et al. (2003)
Vmax, Km[a]	Efficiency of turnover	0.29, 2.7 mM	0.3, 3.2 mM	Woodard et al. (2003)
No. of disulfides[b]	Tertiary structural fidelity	6	6	Ray and Jalili (2011)
Glycosylation site analysis	Location of glycan chain	Asn-77	N/A	Zhang et al. (2010)

[a]Against TAME substrate at pH 8.2
[b]Assay not described

used in peptide mass fingerprinting indicated that a glycan residue was located on a 20 amino acid stretch starting at serine 70 (Zhang et al. 2010). In collaboration with the Gross Lab at Washington University in St. Louis, they came up with a clever method to fragment the peptide further. Using trypsin and pepsin in combination, and increasing the length of time the peptide was exposed to the nonspecific pepsin enzyme, they were able to remove successive amino acids until they could infer that the glycosylated amino acid had been removed (Zhang et al. 2010).

Their findings revealed a somewhat unusual glycosylation. Although the masses of the glycans allowed them to infer a fairly typical distribution of plant glycans, the site of the glycosylation, Asp 77, was atypical. The glycosylated asparagine was followed by serine and asparagine, where, typically, a glycosylated asparagine is followed by any amino acid except proline and then serine or threonine (Bause 1983). The authors confirmed through triple stage mass spectrometry experiments that consensus sequence rules for glycosylation were not followed for bovine trypsin expressed in maize and that the asparagine was N-glycosylated.

In addition to the glycosylation work, additional comparative data for bovine trypsin and the TrypZean™ product were obtained (Ray and Jalili 2011), such as more accurate mass spectrometry data reporting the base molecular weight of TrypZean™ to be 23,294 Da which is only 1 kDa different from the theoretical molecular weight (Table 4.2). They reported that like native bovine trypsin, TrypZean™ has six disulfide bonds. They also report higher specific activity for TrypZean™ than originally reported by Woodard et al. (2003). Since detailed methodology for the activity assays was not presented, it is difficult to know if this higher activity is due to use of differing assay conditions or if the commercial manufacturing process yielded a product with higher specific activity. Regardless, the TrypZean™ product performs similarly to native bovine trypsin in a variety of assays.

4.3.2 Product Development and Early Application Testing

Before the product was named, a product backgrounder was written, and samples of the bovine trypsin purified from corn flour were distributed to a variety of different companies interested in testing it in their processes. ProdiGene trademarked the name TrypZean™ in 2002, rewrote the product backgrounder and, with the help of Sigma-Aldrich, purified larger quantities and distributed the product under the commercial label TrypZean™ later that year. Sigma-Aldrich also formulated the product for cell culture use after carefully testing it in applications with different cell lines.

Dealing directly with customers in the early days of product distribution helped the ProdiGene staff to learn more about how the product was used and often left the scientists scrambling to try and resolve problems that were reported. In one instance, a customer reported that the enzyme had no effect when added to cell culture media and sterile-filtered for use in cell detachment from culture flasks. It

Table 4.3 Detachment times for cell lines with different sources of trypsin

	Porcine trypsin	TrypZean™
Cell line	Time to detach[a]	Time to detach[a]
CRFK (feline kidney)	5 min	5 min
DK (canine kidney)	15 min	15 min
ESK-4 (porcine kidney)	10 min	10 min

[a]Equivalent volumes used for all treatments

was speculated that the larger diameter filter that was used retained most of the small trypsin sample that was supplied. The challenge for scientists at ProdiGene was to then formulate the trypsin for the customers using biosafety cabinets normally used for working with *Agrobacterium*-infected plant tissue. By using a smaller syringe filter and then measuring the activity and making sure it was comparable to that present in cell culture formulated porcine trypsin, the researchers showed that the product worked as expected. Table 4.3 shows that the detachment times were equivalent for the commercially available porcine trypsin/EDTA solution and TrypZean™ formulated in HBSS/EDTA tested against three different cell lines.

In another application, a customer reported that the enzyme did not fully process their protein into the expected form. The customer was using a porcine trypsin product, and it is likely that the porcine trypsin that was used was contaminated with other enzymes such that the highly pure corn-derived trypsin was not able to cleave the target protein to the same extent. Scientists at ProdiGene compared a sample of the TrypZean™ product on SDS-PAGE side by side with a variety of commercially available trypsin products (Fig. 4.1) and found very little intact trypsin in the animal source products compared to corn-derived trypsin shown in lane 3. After adding SDS and boiling, even the highly pure TrypZean™ has some breakdown product, but in none of the cases was the breakdown observed close to what was found in the lanes containing animal source trypsin. It is difficult to know if some of the additional bands present in the animal source products could be other enzymes in addition to degraded trypsin.

Armed with the knowledge that most of the porcine trypsin being used in cell culture applications was very different from the highly purified TrypZean™ product, scientists at ProdiGene were not surprised when customers reported that TrypZean™ was killing cells when used in detachment studies. A few customers reported that when they made the TrypZean™ at the equivalent concentration as the porcine trypsin they were previously using (usually 0.25 %), the cells were detaching immediately and clumping up. The majority of the cells were not viable after treatment with TrypZean™ they formulated themselves. However, once customers were counseled to dilute the TrypZean™ further, they were then able to recover cells normally.

The majority of the feedback received regarding the TrypZean™ product was positive once customers were informed that the corn-derived product might have higher trypsin activity than the porcine trypsin products they normally used. Evidence to support its successful use can be found in many patents and patent

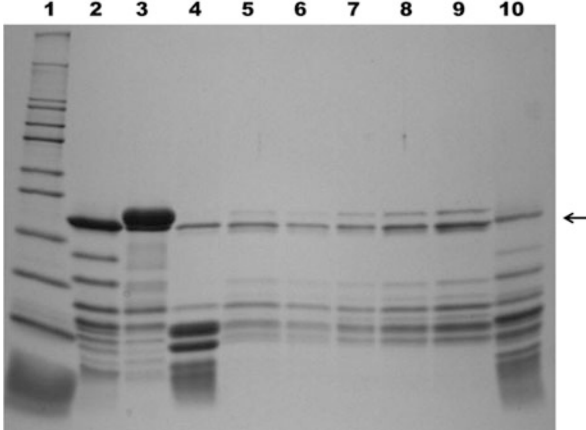

Fig. 4.1 SDS-PAGE of trypsin samples from different sources. Lane 1. Molecular weight markers consisting of 200, 116.3, 97.4, 66.3, 55.4, 36.5, 31.0, 21.5, 14.4, 6.0, and 3.5 kDa standards. Lane 2. 10 μg of bovine trypsin #1. Lane 3. 10 μg of TrypZean™. Lane 4. 10 μg of porcine trypsin #1. Lane 5. 20 μg of porcine trypsin #2. Lane 6. 10 μg of porcine trypsin #2. Lane 7. 10 μg of porcine trypsin #3. Lane 8. 20 μg of porcine trypsin #3. Lane 9. 40 μg of porcine trypsin #3. Lane 10. 10 μg of bovine trypsin #2. The *arrow* shows the band corresponding to intact bovine trypsin. Note that Lanes 7–9 show a porcine trypsin formulated for cell culture use where all of the others are powdered samples

applications that cite the use of TrypZean™ in the manufacture of new vaccines and biopharmaceutical products. The majority of patents found when searching for TrypZean™ in patents are for viral vaccine production where trypsin is used to promote viral infectivity and increase levels of virus production.

Data shown in Fig. 4.2 are a subset of data from a published patent from Crucell Holland B.V. (Pau and UytdeHaag 2003) where corn-derived trypsin was compared to porcine trypsin for the ability to achieve equivalent levels of virus production from a suspension culture of human embryonic PER.C6 cells. The researchers found that about one-tenth the concentration of TrypZean™ was adequate to produce the same concentration of influenza virus (measured in hemagglutinin assays) as when porcine trypsin was used.

Although formulation information is proprietary, the amount of trypsin that is needed in various cell culture applications is reduced compared to that needed when porcine trypsin is used. This difference could help to offset the higher cost of purifying the enzyme from corn using animal component-free materials and processes.

The one anticipated application area where no published data are available is in the bioconversion of precursor proteins into active biotherapeutic proteins and one where the corn-produced enzyme would be expected to perform equivalently to a native bovine trypsin or a recombinant version. At least one biopharmaceutical manufacturer tested the TrypZean™ product in parallel with a microbially produced bovine trypsin in two different bioconversion reactions. They found that the

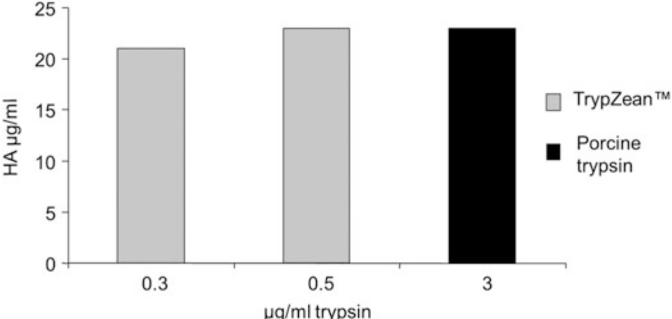

Fig. 4.2 Hemagglutinin concentration produced by Influenza A/Beijing/262/95 grown on Per.C6 cells using different concentrations of TrypZean™ or using the standard amount of porcine trypsin product

two enzymes performed with similar kinetics and product yields (John Howard, personal communication).

4.4 Competing Hosts for Trypsin Expression and Competing Products

4.4.1 Trypsin Expressed in Other Organisms

Recombinant trypsin has been available from a variety of sources for many years. TrypZean™ was developed because of the observation that the supply of recombinant trypsin was limited and rather expensive. The high cost was probably a result of the limited expression levels that could be achieved in host systems or, in the case of *Escherichia coli*, the need to refold the enzyme from inclusion bodies. The first recombinant trypsin made in a foreign host was rat anionic trypsinogen made in mammalian cells (Craik et al. 1985, 1987). The goal of this expression system was to obtain protein for site-directed mutagenesis studies, and, although expression levels were relatively low, the quantities produced were adequate for this need. Rat anionic trypsinogen was later made in *E. coli* (Vasquez et al. 1989) by targeting the enzyme to the periplasm so it would be in soluble form, and approximately 1 mg/L expression was achieved. An approximately equivalent level of expression was reported when trypsin was displayed on the surface of bacteriophage (Corey et al. 1993). Yee and Blanch (1993) improved the level of trypsin expression in the periplasm of *E. coli* using a batch-fed production mode and showed that by delaying induction of trypsin expression until late in the growth phase, they could achieve levels up to 56 mg/L. Others have published similar expression levels when trypsin was targeted to the cytoplasm where the enzyme accumulated in inclusion bodies (Szilagyi et al. 2001, Peterson et al. 2001). Trypsin/trypsinogen expressed in

this way must be resolubilized with a strong chaotrope and then refolded in order to obtain active trypsin. An article by Hohenblum et al. (2004), describing expression of a fusion of part of the T7 bacteriophage promoter sequence to the amino terminus of human trypsinogen, has a good description of the steps needed in order to obtain active trypsin from inclusion bodies. These authors were able to achieve expression levels of up to 200 mg/g of biomass, which is impressive.

Yeast has the advantage of secreting the expressed protein into the medium, minimizing the potential for internal damage to the host. However, expression levels for trypsinogen made in *Pichia pastoris*, a common yeast host, tend to be low and therefore not economically ideal for a product such as trypsin. Hedstrom et al. (1992) demonstrated that trypsinogen expressed in yeast could be made at up to 15 mg/L and then activated to trypsin through enterokinase treatment. Researchers at Eli Lilly published a paper demonstrating that levels of 40 mg/L of bovine trypsinogen could be achieved using this platform if the leader sequence was modified in order to prevent trypsinogen's conversion to trypsin (Hanquier et al. 2003). The only disadvantage to this clever strategy is that it requires a special enzyme to activate the zymogen into active trypsin since autocatalysis is prevented. This nonanimal-sourced enzyme operates at low pH, so further trypsin degradation is minimized. It is likely that the product of the process is used in the manufacture of Eli Lilly's insulin products since trypsin is needed for the conversion of proinsulin to insulin. A patent issued to the biopharmaceutical company, Roche, claims the ability to make trypsin by expressing the enzyme in the zymogen form in *Pichia pastoris* enabled by maintaining a low pH (Muller et al. 2010). This strategy probably helps to maintain the trypsin in inactive form and thereby enable higher levels of expression. Roche markets a high purity trypsin that is used in protein chemistry applications. The product is packaged in small quantities and commands a premium price based on a small application market.

Another expression platform that combines the secretion advantage of *Pichia* with a plant-based expression system is the use of a plant cell culture system for trypsin production. Kim et al. (2011) suggest in a recent publication, in which rice suspension cultures secreted trypsinogen out of the cell, that this production platform might be more economical due to the fact that the protein is secreted into the medium where it comprises up to 7.5 % of total protein. Similar to bovine trypsinogen expressed in maize (Woodard et al. 2003), the cell culture product was in active (trypsin) form, and although the authors did not purify the active trypsin product, they compared it to purified native bovine trypsin as well as that purified from maize on Western blots and zymograms, where the trypsin from rice showed similar patterns to the maize-derived trypsin. It remains to be seen if this platform can produce a cost-effective product since other proteolytic enzymes as well as polysaccharides, which can complicate recovery and purification, tend to accumulate in plant cell culture media (Hellwig et al. 2004). In addition, a substantial fraction of some proteins secreted into cell culture media are lost through adsorption on the surface of vessels, impacting protein recovery (Doran 2006; Kwon et al. 2012).

Despite the promise of many platforms for making an alternative source of animal-derived trypsin, very few manufacturers sell recombinant trypsin at a cost that is competitive with maize-derived trypsin. Bovine trypsin made in maize sells for about $10/mg. There are a few vendors currently selling trypsin made from *E. coli* for about the same price, but the *Pichia*-derived enzymes are about twice as expensive (Internet search conducted on 27 October 2012). None of these platforms can compete as yet with the low cost of bovine or porcine pancreatic trypsin, where the enzyme is extracted in crude form from waste organs and sells for a few hundred dollars per gram. In contrast to this is the extremely high cost that is required to make the product in mammalian cells where the one example found for recombinant human trypsin costs $5,200 for 1 mg of enzyme made in Chinese Hamster Ovary cells by Prospec Bio, Israel (Internet search conducted 27 October 2012).

4.4.2 Competing Products and Platforms

Although recombinant trypsin made from other sources does not appear to compete to a large extent with the corn-derived product, there are other products that compete directly or indirectly with TrypZean™ in applications. Direct competitors include trypsin from nonmammalian sources as well as trypsin-like enzymes. Accutase® is from an aquatic organism and includes proteolytic and collagenase activities. It is unique in that it is active at lower temperatures and can be inactivated by incubation at 37 °C. Another replacement enzyme product is the TrypLE™ line of products by Life Technologies. These products are based on a trypsin-like enzyme from *Fusarium oxysporum*, originally called rProtease (Gibco) and rebranded (possibly with manufacturing changes) in 2003 or 2004. TrypLE™ claims advantages over porcine trypsin, such as room-temperature stability and the fact that protease inhibitor is not needed to quench TrypLE's activity after cell detachment from substrate.

Another area of competition is replacement technologies that would obviate the need for trypsin. An example of this is the increasing use of suspension cell lines whose growth is not dependent on their attachment to surfaces. One popular suspension cell line is the PER.C6 cells mentioned earlier for growing influenza virus (Pau et al. 2001). EB66 cells of duck origin are also growing in popularity for a variety of applications where adherent cell lines would normally be used (Brown and Mehtali 2010). Adoption of the use of these cell lines means that trypsinization is no longer needed for cell passage. Another growing trend in cell culture is the use of microcarrier beads to increase the surface area upon which adherent cells can grow. Microcarriers allow cells to grow to higher densities, as though they were suspension cells. The use of microcarriers still requires trypsinization for cell passage, however, and if the product is a therapeutic protein, an animal source free trypsin will be desirable.

4.5 Public Acceptance and Mainstream Adoption

Hurdles to public acceptance of an alternative trypsin product are low. Fears of contaminated vaccines and potentially fatal bovine-derived pathogens such as TSE outweigh the perceived risk of genetically modified organisms. Evidence of mainstream adoption is the mention of TrypZean™ in a basic textbook on cell culture (Freshney 2010) and in a compilation on regenerative medicine (Allan and Strunk 2012). Several independent groups have demonstrated the efficacy and comparability of TrypZean™ to bovine trypsin and other recombinant trypsins available in the market. In the few publications where TrypZean™ and TrypLE™ have been tested side by side, the two products appear to perform equally well (Rourou et al. 2009; Carvalho et al. 2011).

TrypZean™ seems particularly favored by researchers performing artificial insemination studies and in stem cell work. Artificial insemination of cows with bull semen with undetectable levels bovine herpes virus 1 (BHV-1) resulted in transmission of the virus to a BHV-1 free herd (Bielanski et al. 1988). Because this infection can result in abortion and infertility in cows, Bielanski and colleagues tested the ability of porcine trypsin to inactivate BHV-1 in bovine semen. This work demonstrated that trypsin treatment was effective at inactivating BHV-1 and results in viable embryos in recipients of the treated semen. More recently, researchers have demonstrated that in vitro-derived bovine embryos can be treated with trypsin to inactivate BHV-1 (Seidel et al. 2007). This group favored the TrypZean product to a "trypsin-like" enzyme, but this work is only reported in an abstract. Mattson et al. (2008) demonstrated that there was no significant difference in measures of fertility and embryo development between porcine trypsin- and TrypZean™-treated bovine sperm. The procedure used was a combination of trypsin treatment followed by density gradient centrifugation which were important for motility and fertilization.

The growing field of stem cell use in regenerative medicine, where trypsin is used in the production of stem cells, is also benefitting from the availability of animal-free sources of trypsin. TrypZean™ has been used to aid in the isolation of multipotent epithelial cells from placental amnion membrane (Murphy et al. 2010). Carvalho et al. (2011) demonstrated that comparable adipose-derived stromal stem cells resulted regardless of which trypsin product was used to harvest these cells: porcine-derived trypsin, TrypZean™, or TrypLE Express™. TrypZean™ was also used recently to harvest human bone marrow mesenchymal stromal cells produced on a large scale under cGMP conditions (Fekete et al. 2012). A recent patent application by Silva and Gonzalez (2013) cites the use of TrypZean™ in the subculture of stem cells isolated from the lining of umbilical cords. With the progress being made in stem cell research, it is expected that the demand for alternative sources of trypsin will grow when clinical grade stem cells need to be made for patient use.

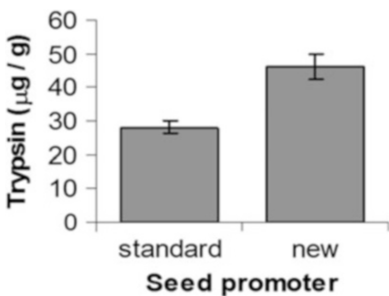

Fig. 4.3 Expression level of trypsin (on a dry weight basis) per seed for original trypsinogen construct targeted to embryo and the second-generation trypsinogen embryo-targeted construct

4.6 Possible Strategies for Producing Higher Levels of Bovine Trypsin in Transgenic Maize Seed

4.6.1 Use of Different Promoters

The current TrypZean™ product is made with first-generation seed as described in Woodard et al. (2003). Many improvements have been made in the expression platform and in the plant biotechnology field over the past 10 years but have not been implemented for trypsin expression. Some of the improvements are described in subsequent publications from members of the former ProdiGene research group (Hood et al. 2007, 2012; Streatfield et al. 2010; Hayden et al. 2012; Egelkrout et al. 2013). Work was initiated at ProdiGene with the goal of increasing trypsin expression through the use of new promoters and targeting sequence combinations and carried on subsequently at other places. One improvement was made through use of a full-length globulin promoter instead of a truncated version resulting in a more than 50 % increase in the levels of trypsin (see Fig. 4.3).

It is possible that other seed storage promoters similar to the globulin promoter might be a better choice for the trypsin product, depending upon kinetics of protein accumulation and location in the seed. One of the advantages of using seed storage protein promoters is that these genes are active late in seed development. This delay in protein accumulation may help with trypsin expression as delayed induction of trypsin increased overall yield in a microbial platform (Yee and Blanch 1993). Scientists at Ventria Bioscience almost doubled the expression of lysozyme in rice by combining two different expression cassettes, one using their conventional rice *Glutelin 1* promoter and signal peptide (Huang et al. 2002) with another using a wheat *puroindoline b* promoter and signal peptide. Although puroindoline b is not a storage protein, the use of this promoter targeted lysozyme to endosperm protein bodies (Hennegan et al. 2005). Combining an endosperm targeting promoter cassette for trypsinogen with the maize globulin embryo-targeting promoter cassette for trypsinogen might produce a similar enhancement.

4.6.2 Other Strategies

Any strategy commonly used to increase the expression of heterologous genes in transient or transgenic plant platforms are worth exploring here as well. The use of strategies that result in increasing transcription or translation or stabilizing the products of these processes is a potential strategy that might boost trypsin/trypsinogen expression. Excellent reviews by Streatfield (2007), Desai et al. (2010), and Egelkrout et al. (2012) describe the numerous strategies that are used to boost expression in transgenic, nuclear-transformed, and transient plant expression platforms. One recent addition to this list is the use of double terminators as described in Beyene et al. (2011) for a transiently expressed reporter gene in sugarcane. Although there is no evidence suggesting that transgene silencing is limiting trypsin expression in the current transgenic platform, it would be interesting to see if double terminators helped to boost expression for stably integrated genes.

A recent paper by Shigemitsu et al. (2012) demonstrated that the use of RNAi targeting rice storage proteins decreased levels of the targeted proteins and may have helped to boost the level of human growth hormone that was targeted to protein bodies in the endosperm. Since a reference without RNAi was not included, it is difficult to know if expression was actually increased. It seems likely however that the expression product competes for room with storage proteins, and this strategy may be one of a combination of strategies that help to boost expression in proteins targeted to seed.

One strategy that is likely to help boost trypsin expression in maize is one where a trypsin-specific protease inhibitor that could help to offset the detrimental effects of trypsin is co-expressed in the same compartment of the seed (Goulet et al. 2010). As long as the two expression products could be separated during purification, this strategy could offer the ability to increase trypsin levels above whatever limitation the plant host has for this detrimental enzyme. This strategy would augment the benefit that is already realized due to the presence of endogenous corn trypsin inhibitor. Likewise, the presence of an additional trypsin inhibitor could help to keep trypsin inactive during extraction.

4.7 Concluding Remarks

In the past decade, animal-origin free reagents have gained wide acceptance in the marketplace. In 2003, TrypZean™ was a novel product, and many companies have since tested its use in their biopharmaceutical manufacturing processes. The many papers, patents, and patent applications that have cited the use of TrypZean™ are testament to its effectiveness. Now, competing products have entered the marketplace and may continue to do so as advances are made in other or similar platforms. Competing products have been tested side by side with TrypZean™ in some applications. The majority of the works cited here found equivalent performance

when TrypZean™ was tested against its primary competitor, TrypLE™, in cell culture applications. In such cases, the decision to adopt one or the other in a process will be based on cost and reliability of the product supply. Maintaining a strong place in the market relies on assuring that the product is manufactured to a consistent quality, the pricing remains competitive with alternative products, and that there is a stable supply. Any improvements that could be made to increase the expression of trypsin in maize lines or reduce the cost of purifying the enzyme could result in cost savings that, if passed along to consumers, would help to ensure a strong future for this product.

References

Allan DS, Strunk D (2012) Regenerative therapy using blood-derived stem cells. Springer, New York

Bause E (1983) Structural requirements of N-glycosylation of proteins. Biochem J 209:331–336

Beyene G, Buenrostro-Nava MT, Damaj MB, Gao S-J, Molina J, Mirkov TE (2011) Unprecedented enhancement of transient gene expression from minimal cassettes using a double terminator. Plant Cell Rep 30:13–25

Bielanski A, Loewen KG, Hare WCD (1988) Inactivation of bovine herpesvirus-1 (BVH-1) from in vitro infected bovine semen. Theriogenology 30:649–657

Brown SW, Mehtali M (2010) The avian EB66 cell line, application to vaccines, and therapeutic protein production. PDA J Pharm Sci Technol 64:419–425

Carvalho PP, Wu X, Yu G, Dietrich M, Dias IR, Gomes ME, Reis RL, Gimble JM (2011) Use of animal protein-free products for passaging adherent human adipose-derived stromal/stem cells. Cytotherapy 13:594–597

Corey DR, Shiau AK, Yang Q, Janowski BA, Craik CS (1993) Trypsin display on the surface of bacteriophage. Gene 128:129–134

Craik CS, Largman C, Fletcher T, Roczniak S, Barr PJ, Fletterick R, Rutter WJ (1985) Redesigning trypsin: alteration of substrate specificity. Science 228:291–297

Craik CS, Roczniak S, Largman C, Rutter WJ (1987) The catalytic role of the active site aspartic acid in serine proteases. Science 237:909–913

Davie EW, Neurath H (1955) Identification of a peptide released during autocatalytic activation of trypsinogen. J Biol Chem 212:515–529

Desai PN, Shrivastava N, Padh H (2010) Production of heterologous proteins in plants: strategies for optimal expression. Biotechnol Adv 28:427–435

Doran PM (2006) Loss of secreted antibody from transgenic plant tissue cultures due to surface adsorption. J Biotechnol 122:39–54

Egelkrout E, Rajan V, Howard JA (2012) Overproduction of recombinant proteins in plants. Plant Sci 184:83–101

Egelkrout E, McGaughey K, Keener T, Ferleman A, Woodard S, Devaiah S, Nikolov Z, Hood EE, Howard J (2013) Enhanced expression levels of cellulase enzymes using multiple transcription units. Bioenergy Res 6:699–710

Evangelista RL, Kusnadi AR, Howard JA, Nikolov ZL (1998) Process and economic evaluation of the extraction and purification of recombinant β-glucuronidase from transgenic corn. Biotechnol Prog 14:607–614

Fekete N, Gadelorge M, Furst D, Maurer C, Dausend J, Fleury-Cappellesso S, Mailander V, Lotfi R, Ignatius A, Sensebe L, Bourin P, Schrezenmeier H, Rojewski MT (2012) Platelet lysate from whole blood-derived pooled platelet concentrates and apheresis-derived platelet concentrates for the isolation and expansion of human bone marrow mesenchymal stromal

cells: production process, content and identification of active components. Cytotherapy 14:540–554
Freshney RI (2010) Culture of animal cells: a manual of basic technique and specialized applications. Wiley, Hoboken, NJ
Goulet C, Benchabane M, Anguenot R, Brunelle F, Khalf M, Michaud D (2010) A companion protease inhibitor for the protection of cytosol-targeted recombinant proteins in plants. Plant Biotechnol J 8:142–154
Hanquier J, Sorlet Y, Desplancq D, Baroche L, Ebtinger M, Lefevre J-F, Pattus F, Hershberger CL, Vertes AA (2003) A single mutation in the activation site of bovine trypsinogen enhances its accumulation in the fermentation broth of the yeast *Pichia pastoris*. Appl Environ Microbiol 69:1108–1113
Hayden CA, Egelkrout EM, Moscoso AM, Enrique C, Keener TK, Jimenez-Flores R, Wong JC, Howard JA (2012) Production of highly concentrated, heat-stable hepatitis B surface antigen in maize. Plant Biotechnol J 10:979–984
Hedstrom L, Szilagyi L, Rutter WJ (1992) Converting trypsin to chymotrypsin: the role of surface loops. Science 255:1249–1253
Hellwig S, Drossard J, Twyman RM, Fischer R (2004) Plant cell cultures for the production of recombinant proteins. Nat Biotechnol 22:1415–1422
Hennegan K, Yang D, Nguyen D, Wu L, Goding J, Huang J, Guo F, Huang N, Watkins SC (2005) Improvement of human lysozyme expression in transgenic rice grain by combining wheat (*Triticum aestivum*) puroindoline b and rice (*Oryza sativa*) Gt1 promoters and signal peptides. Transgenic Res 14:583–592
Hohenblum H, Vorauer-Uhl K, Katinger H, Mattanovich D (2004) Bacterial expression and refolding of human trypsinogen. J Biotechnol 109:3–11
Hood EE, Woodard SL (2002) Industrial proteins produced from transgenic plants. In: Hood EE, Howard JA (eds) Plants as factories for protein production. Kluwer, Dordrecht, pp 119–135
Hood EE, Love R, Lane J, Bray J, Clough R, Pappu K, Drees C, Hood KR, Yoon S, Ahmad A (2007) Subcellular targeting is a key condition for the high-level accumulation of cellulase protein in transgenic maize seed. Plant Biotechnol J 5:709–719
Hood EE, Devaiah SP, Fake G, Egelkrout E, Teoh TK, Requesens DV, Hayden C, Hood KR, Pappu KM, Carroll J, Howard JA (2012) Manipulating corn germplasm to increase recombinant protein accumulation. Plant Biotechnol J 10:20–30
Howard JA, Hood EE (2000) Commercial production of proteases in plants. US 6,087,558 & US 7,049,484 B2
Huang J, Nandi S, Wu L, Yalda D, Bartley G, Rodriguez R, Lonnerdal B, Huang N (2002) Expression of natural antimicrobial human lysozyme in rice grains. Mol Breed 10:83–94
Huber R, Bode W (1978) Structural basis of the activation and action of trypsin. Acc Chem Res 11:114–122
Keil B (1971) Trypsin. In: Boyer PD (ed) The enzymes, vol 82. Academic, New York, pp 249–275
Kemmler W, Peterson JD, Steiner DF (1971) Studies on the conversion of proinsulin to insulin. I. Conversion in vitro with trypsin and carboxypeptidase B. J Biol Chem 246:6786–6791
Kim N-S, Yu H-Y, Chung N-D, Shin Y-J, Kwon T-H, Yang M-S (2011) Production of functional recombinant Bovine trypsin in transgenic rice cell suspension cultures. Protein Expr Purif 76:121–126
Kusnadi AR, Nikolov ZL, Howard JA (1997) Production of recombinant proteins in transgenic plants: practical considerations. Biotechnol Bioeng 56:473–484
Kwon J-Y, Lee K-H, Cheon S-H, Ryu H-N, Kim SJ, Kim D-I (2012) Adsorptive loss of secreted recombinant proteins in transgenic rice cell suspension cultures. Plant Cell Rep 31:551–560
Liepnieks JJ, Light A (1974) Preparation of beta-trypsin by affinity chromatography of enterokinase-activated bovine trypsinogen. Anal Biochem 60:395–404
Mattson KJ, Devlin BR, Loskutoff NM (2008) Comparison of a recombinant trypsin with the porcine pancreatic extract on sperm used for the in vitro production of bovine embryos. Theriogenology 69:724–727

McClenahan SD, Krause PR, Uhlenhaut C (2011) Molecular and infectivity studies of porcine circovirus in vaccines. Vaccine 29:4745–4753

Merten O-W (2002) Virus contamination of cell cultures—a biotechnological view. Cytotechnology 39:91–116

Muller R, Glaser S, Geipel F, Thalhofer J-P, Rexer B, Schneider C, Ratka M, Ronning S, Eckstein H, Giessel C (2010) Method for producing recombinant trypsin. US 7,666,629

Murphy S, Rosli S, Acharya R, Mathias L, Lim R, Wallace E, Jenkin G (2010) Amnion epithelial cell isolation and characterization for clinical use. Curr Protoc Stem Cell Biol 13:1E.6.1–1E.6.25

Nikolov Z, Hammes D (2002) Production of recombinant proteins from transgenic crops. In: Hood EE, Howard JA (eds) Plants as factories for protein production. Kluwer, Dordrecht, pp 159–174

Pau MG, UytdeHaag AG (2003) Use of recombinant trypsin for vaccine production. WO 03/076462 A1

Pau MG, Ophorst C, Koldijk MH, Schouten G, Mehtali M, Uytdehaag F (2001) The human cell line PER.C6 provides a new manufacturing system for the production of influenza vaccines. Vaccine 19:2716–2721

Peterson FC, Gordon NC, Gettins PGW (2001) High-level bacterial expression and ^{15}alanine-labeling of bovine trypsin. Application to the study of trypsin-inhibitor complexes and trypsinogen activation by NMR spectroscopy. Biochemistry 40:6275–6283

Polak-Vogelzang AA, Angulo AF, Brugman J, Reijgers R (1990) Survival of *Mycoplasma hyorhinis* in trypsin solutions. Biologicals 18:97–101

Rawlings ND, Barrett AJ (1994) Families of serine peptidases. In: Barrett AJ (ed) Methods in enzymology, vol 244. Academic, San Diego, CA, pp 19–61

Ray K, Jalili PR (2011) Characterization of TrypZean: a plant based alternative to bovine-derived trypsin. Biopharm Int 24(10):44–48

Rogers JC (1985) Two barley alpha-amylase gene families are regulated differently in aleurone cells. J Biol Chem 260:3731–3738

Rourou S, van de Ark A, van de Velden T (2009) Development of an animal-component free medium for Vero cells culture. Biotechnol Prog 25:1752–1761

Schroeder DD, Shaw E (1968) Chromatography of trypsin and its derivatives. Characterization of a new active form of bovine trypsin. J Biol Chem 243:2943–2949

Seidel GE Jr, Turk ML, Gordy PW, Bowen RA (2007) Recombinant bovine trypsin made in maize inactivates bovine herpes virus-1 absorbed to the bovine zona pellucida. Reprod Fertil Dev 19:236 [abstract]

Shigemitsu T, Ozaki S, Saito Y, Kuroda M, Morita S, Satoh S, Masumura T (2012) Production of human growth hormone in transgenic rice seeds: co-introduction of RNA interference cassette for suppressing the gene expression of endogenous storage proteins. Plant Cell Rep 31:539–549

Silva FJ, Gonzalez R (2013) Umbilical cord lining stem cells and methods and material for isolating and culturing same. US2013/0065302

Streatfield SJ (2007) Approaches to achieve high-level heterologous protein production in plants. Plant Biotechnol J 5:2–15

Streatfield SJ, Bray J, Love RT, Horn ME, Lane JR, Drees CF, Egelkrout EM, Howard JA (2010) Identification of maize embryo-preferred promoters suitable for high-level heterologous protein production. GM Crops 1:162–172

Stryer L (1995) Biochemistry, 4th edn. WH Freeman, New York

Szilagyi L, Kenesi E, Katona G, Kaslik G, Juhasz G, Graf L (2001) Comparative *in vitro* studies on native and recombinant human cationic trypsins. J Biol Chem 276:24574–24580

Vasquez JR, Evnin LB, Higaki JN, Craik CS (1989) An expression system for trypsin. J Cell Biochem 39:265–276

Woodard SL, Mayor JM, Bailey MR, Barker DK, Love RT, Lane JR, Delaney DE, McComas-Wagner JM, Mallubhotla HD, Hood EE, Dangott LJ, Tichy SE, Howard JA (2003) Maize-

derived bovine trypsin: characterization of the first large-scale, commercial protein product from transgenic plants. Biotechnol Appl Biochem 38:123–130
Yee L, Blanch HW (1993) Recombinant trypsin production in high cell density fed-batch cultures in Escherichia coli. Biotechnol Bioeng 41:781–790
Zhang H, Huang RY-C, Jalili PR, Irungu JW, Nicol GR, Ray KB, Rohrs HW, Gross ML (2010) Improved mass spectrometric characterization of protein glycosylation reveals unusual glycosylation of maize-derived bovine trypsin. Anal Chem 15:10095–10101

Chapter 5
Production of Pharmaceutical Grade Recombinant Native Aprotinin and Non-oxidized Aprotinin Variants Under Greenhouse and Field Conditions

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5.1 Introduction

5.1.1 Aprotinin Product Background

Aprotinin is a 58-amino acid active serine protease inhibitor of bovine origin that is processed from a preproprotein precursor (Laskowski and Kato 1980). The active protein conformation requires three disulfide bridges and appropriate processing from both N- and C-terminal prepropeptides to form the 6,511 Da product. Aprotinin for pharmaceutical application has been historically purified using standard chromatography procedures from bovine lung tissues isolated from specialized bovine herds from regions devoid of bovine spongiform encephalopathy occurrence. Aprotinin has been explored for clinical applications for four decades for a variety of clinical applications (Beierlein et al. 2005). Bayer HealthCare Pharmaceuticals' Trasylol®, natural aprotinin, is an FDA-approved product indicated for prophylactic use to reduce perioperative blood loss and the need for blood transfusion in patients undergoing cardiopulmonary bypass in the course of coronary

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artery bypass graft surgery (CABG; Munoz et al. 1999; Sedrakyan et al. 2004). The drug, manufactured from residual bovine lung materials, was approved in the United States in 1993. However, recent international studies have indicated increased risk of in-hospital death and 5-year mortality rates among aprotinin recipients when compared with non-recipients (Mangano et al. 2006, 2007). In late 2008, Bayer HealthCare announced that marketing of the product was temporarily suspended pending review of additional clinical studies (Stamou et al. 2009). In spite of the adverse events associated with the drug in CABG patients, clinical studies continue to explore the application of aprotinin in other indications, both prophylactic and therapeutic, where the control of pathophysiological inflammatory cascades is desirable (Maffulli et al. 2008; Orchard et al. 2008; Rademakers et al. 2009). Further, the Nordic Group obtained the rights to Trasylol® following the opinion issued by the Committee for Medicinal Products for Human Use of the European Medicines Agency in February 2012, recommending that the marketing authorization for aprotinin to be reinstated in the European Union. The recommendation comes after a full review of the benefits and risks of all antifibrinolytic medicines which found the results of the studies noted above, which led to the suspension of aprotinin, were unreliable (Nordic Pharma Group 2012). These developments and the ongoing studies suggest that the market for aprotinin could expand once again provided an alternative active pharmaceutical ingredient (API) to bovine tissue could be more reliably produced without raising concerns over animal-associated adventitious agents, such as bovine spongiform encephalopathy prions.

5.1.2 Recombinant Protein Expression in Plants

Plants have historically been a source for medicinal active ingredients. More than four billion people utilize plant-derived products to meet their primary health care (Farnsworth et al. 1985; Dias et al. 2012). There are ~120 distinct drugs derived from plants representing close to 70 % of the approved drugs in the past 20 years (Taylor 1996; Newman and Cragg 2007). Plants offer agricultural scale with associated cost advantages, and many groups since the 1980s have sought to use plants as sources of diversify medicinal products (Sharma and Sharma 2009; Sourrouille et al. 2009). Due to their eukaryotic protein processing and established success surrounding agricultural products, plants are viewed as an attractive alternative production system for many biologics (Floss et al. 2007; Lico et al. 2008; Ma et al. 2003; Plasson et al. 2009; Pogue et al. 2010). The recent approval of Protalix Biotherapeutic's Elelyso (taliglucerase alfa) by the US Food and Drug Administration (Maxmen 2012) represents the first approved plant-produced human biologic. This product, produced in transgenic carrot cells, possesses benefits over other cell-based systems but still requires capital intensive production methods, requiring multiplicative costs for increased scale (Odum 2001). Nevertheless, Elelyso's approval establishes that plants can meet the stringent demands for

human products, at competitive scale and costs. Indeed, Protalix sells Elelyso at 75 % of the price of Cerezyme, the leading product in this market sector (Maxmen 2012).

Agriculture offers several advantages as a biologic production system. Plants allow capital-efficient design of upstream manufacturing capacity at various scales providing cost savings that cannot be easily matched by fermentation technologies. Considerable capital and time are required to construct the upstream facilities for cell culture production (Thiel 2004). The upstream facility must be linked with downstream capabilities supporting product purification and characterization (Thiel 2004; Pogue et al. 2002). Agriculture-based production requires less specialized upstream facilities such as climate-controlled growth chambers, linked with similar downstream production capabilities. The use of plants therefore reduces capital expenditures and often provides for efficient purification of products (Pogue et al. 2002, 2010).

Traditional transgenic approaches were initially pursued for transgenic production of recombinant proteins (Hiatt et al. 1989; Fischer et al. 2003). Plant-based virus systems were rapidly adapted as plant expression tools. The rapid replication cycle of the virus systems provided amplification of messenger RNA and the resulting proteins provide for a "burst" of recombinant expression that can provide impressive yields (reviewed in Pogue et al. 2010; Pogue and Holzberg 2013).

5.2 Plant Production Strategies

5.2.1 Transgenic Plants

We have explored the expression of aprotinin in plants from both the literature and experimental perspectives and can offer it as an example of a commercial product produced from plant-based sources. Aprotinin has been viewed as a model protein for plant protein expression due to its small size, simple structure, and lack of glycosylation. To date, several groups have expressed and purified recombinant bovine aprotinin (r-aprotinin) from transgenic plant materials. Often groups will use crude yields of proteins to compare the efficacy of the varied expression systems. For example, the crude yields of r-aprotinin varied per system: 0.17 % total protein in the corn seed (Azzoni et al. 2002; Zhong et al. 2007), 0.65 mg/L plant media or 3.7 % of secreted protein present in Spirodela (duckweed) growth media (Rival et al. 2008), and 0.5 % total soluble protein in selected leaves in transplastomic tobacco (Tissot et al. 2008). These measurements of yield are difficult to compare due to the vastly different levels of protein present in the various targeted tissues or the efficiencies in extraction from these tissues. Production of r-aprotinin in transgenic corn seed illustrates this point well. When the entire corn seed is extracted, the total soluble protein concentration is much lower compared with extraction of just the germ (Zhong et al. 2007). Optimized extraction methods and selective

extraction from the germ resulted in a >10-fold increase in recovered r-aprotinin activity from corn seed germ (Zhong et al. 2007). These results illustrate the critical nature of downstream processing efficiencies to ensure yield and quality of purified protein product (Plesha et al. 2009; Zhong et al. 2007). The purified aprotinin showed comparable protein size and trypsin inhibitory activity as control aprotinin (Rival et al. 2008; Tissot et al. 2008; Zhong et al. 2007). Unfortunately, pharmaceutically relevant analyses of the identity, purity, and potency of the aprotinin were not performed.

In spite of the touted crude yields, the only manner of visualization or detection of the aprotinin is presented in terms of immunoassays or activity assays in the studies cited above. Standard purity or size visualization of product in crude plant lysates by protein gel analysis was not provided. In contrast to transgenic expression strategies, transient plant expression vectors generally offer higher yield potential enabling product analysis in direct plant lysates and development of appropriate product release tests. Aprotinin serves as a promising product candidate well suited for transient plant expression.

5.2.2 Transient Expression

GENEWARE® is a plant virus-based expression system that allows manipulation of an RNA virus genome in cDNA form, generation of inoculum through in vitro-derived RNA transcripts, and plant inoculation with transcripts or virions derived from a packing host (Fig. 5.1). The transfected plants are grown in greenhouses or open fields, harvested, and subjected to protein extraction, purification, and formulation (Fig. 5.1). GENEWARE® is composed of a hybrid replicon derived from tobacco mosaic *Tobamovirus* (TMV), principally strains U1 (replication and movement proteins) and U5 (coat protein (CP) and 3′ untranslated region). The viral proteins involved in RNA replication are directly transcribed from the genomic RNA, whereas expression of internal genes is through the production of subgenomic RNAs (Pogue et al. 1998, 2002; Fig. 5.1). The production of subgenomic RNAs is controlled by sequences in the *Tobamovirus* genome that function as subgenomic promoters. The CP is translated from a subgenomic RNA and is the most abundant protein and RNA produced in the infected cell (Pogue et al. 2002; Pogue and Holzberg 2013). GENEWARE® expression system takes advantage of independent virus functions, including cell-to-cell and systemic movement activities mediated by movement protein (MP) and CP, respectively. GENEWARE® also exploits the ability of the viral subgenomic promoter's activity to reprogram the translational priorities of the plant host cells so that virus-encoded proteins are synthesized at similar high levels as the TMV CP (Shivprasad et al. 1999; Pogue et al. 2010). A foreign gene encoding the protein for overexpression is added in place of the virus CP so it will be expressed from the

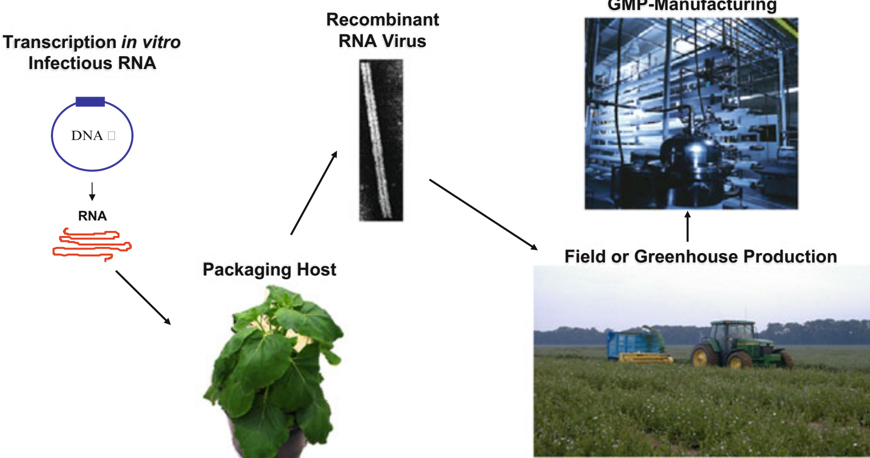

Fig. 5.1 The GENEWARE® expression system. Gene sequences are introduced into a plasmid containing the virus cDNA downstream of the native TMV U1 coat protein subgenomic promoter and upstream of the U5 subgenomic promoter, coat protein gene, and 3′ non-translated region. RNA transcripts are produced in vitro using T7 RNA polymerase and are used as inoculum on a packaging host (*Nicotiana benthamiana*—Nb). Recombinant virions are isolated from the packaging host and tested for intact gene encoding recombinant protein using genetic and functional tests. These are then mass inoculated on Nb grown in greenhouses or *Nicotiana excelsiana* grown in the field (shown in figure). Transfected plant tissue is harvested in mass, and proteins are extracted and purified in a facility capable of cGMP manufacturing

endogenous virus CP promoter. A second CP promoter of lower transcriptional strength, divergent in sequence from the endogenous (TMV U1) CP promoter, is placed downstream of the heterologous coding region, and a virus CP gene is then added (Pogue and Holzberg 2013). This encodes a third subgenomic RNA allowing the virus vector to express all requisite genes for virus replication and systemic movement in addition to the heterologous gene intended for overexpression (Shivprasad et al. 1999; Pogue et al. 2002). GENEWARE® vectors infect various tobacco-related species (genus *Nicotiana*), including *tabacum*, *benthamiana*, and a KBP-proprietary *Nicotiana* hybrid species, *Nicotiana excelsiana* (Fitzmaurice 2002; Pogue et al. 2010). The infectious vector RNA enters plant cells via wounds induced by an abrasive material. The virus replicates in the initial cell, moves to adjacent cells to produce round infection foci, and then enters the plant's vascular system for transport to aerial leaves. There it systemically infects the majority of cells in each infected leaf, and the foreign gene is expressed in all cells that express other viral protein products, including replicase, MP, and CP (Fig. 5.1; Pogue et al. 2002, 2010).

MGKMASLFATFLVVLVSLSLASESSARPDFCLEPPYTGPCKARIIRYFYNAKAGLCQTFVY

GGCRAKRNNFKSAEDCMRTCGGA

Fig. 5.2 The r-aprotinin expression construct is described as deduced amino acid sequence. The modified aprotinin gene sequence is shown with *Nicotiana* extensin signal peptide underlined and mature aprotinin not underlined. The DNA sequence encoding the synthetic aprotinin gene was constructed using codon biases based on the tobacco mosaic virus coat protein sequence (not shown)

5.3 Production of r-Aprotinin Using Transient Virus Systems

5.3.1 Small-Scale Expression Strategy

A synthetic cDNA of the mature bovine aprotinin gene was constructed as an in-frame fusion with the *Nicotiana benthamiana* (Nb) extensin signal peptide (Pogue et al. 2010; Fig. 5.2). The aprotinin gene was subcloned into the TMV-based GENEWARE® vector under the control of the T7 RNA polymerase promoter to produce expression plasmid construct pKBP2602. RNA transcripts were prepared and inoculated on Nb plants. Characteristic viral symptoms were noted ~6–12 days postinoculation. Based on extensive experimentation including the testing of inoculum amounts, age of plant, timing of infection, and harvest, the optimized production conditions were determined for r-aprotinin to be 14 days postinoculation. Leaf and stem materials, generally above the inoculated leaves, were homogenized, acidified, and clarified using centrifugation to remove plant membranes and photosynthetic proteins (Fig. 5.3). The r-aprotinin expressed in plants co-migrated with the Trasylol® control under SDS-PAGE conditions (Fig. 5.3). Further, the molecular mass of r-aprotinin in the clarified homogenate of 6,512 Da, as determined by matrix-assisted laser desorption–ionization time-of-flight (MALDI-TOF) mass spectrometry, matched that of the control (Table 5.1). Finally, using trypsin inhibition assays, significant inhibition of serum protease activity was also determined in the crude extract, and the purified protein showed comparable inhibition activity to native bovine aprotinin, ~7,100 trypsin inhibitory units (TIU) per mg of extract protein (Table 5.1; Fritz and Wunderer 1983).

5.3.2 Large-Scale Production

For large-scale manufacturing of r-aprotinin, Nb plants grown under greenhouse conditions or those in open field cultivation (*N. excelsiana*; Fig. 5.1) were used as production hosts. Recombinant TMV virions were isolated from plants infected with RNA transcripts derived from pKBP2602 plasmid DNA. These virions were

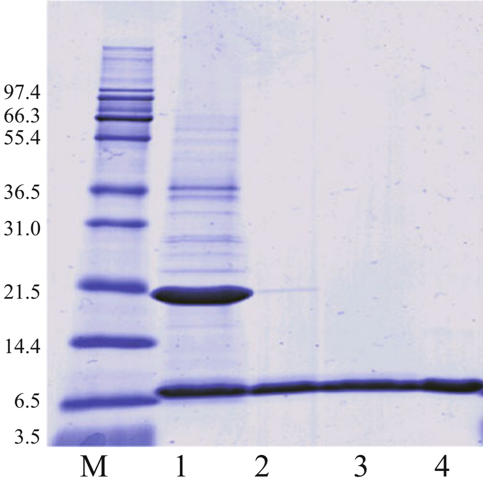

Fig. 5.3 Expression and purification of r-aprotinin from *Nicotiana* plants and comparison with Trasylol®. Virion preparations containing tobacco mosaic virus (TMV)-expression vector encoded by plasmid pKBP2602 were inoculated on *Nicotiana excelsiana* plants. Plants were harvested 14 days postinoculation. Lane 1 shows clarified *Nicotiana* extract with prominent r-aprotinin band and the TMV coat protein. Eluents from ion chromatography and RP-HPLC are shown in lanes 2 and 3. Trasylol 1® is loaded in lane 4 for comparison. Molecular weight markers containing known molecular weight proteins are loaded at right. Proteins were analyzed using 4–12 % Bis-Tris sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and subjected to Coomassie Brilliant Blue staining

shown to possess RNA genomes with complete aprotinin reading frames by sequence and functional analysis. A spray inoculation method was used for mass plant inoculations where virions were mixed with an abrasive material applied to plants under high pressure. This creates simultaneous injury and virion delivery in leaf tissues. In general, plants were bulk harvested 14 days postinfection. A similar strategy for extraction at large-scale clarification was used as small scale described above. Recovered plant materials were homogenized and clarified using treatment for reduction in pH and centrifugation (Fig. 5.3). The protein fraction containing the r-aprotinin was concentrated using ultrafiltration. Cation exchange chromatography was used as the first separation step followed by reverse-phase chromatography. The final r-aprotinin product was concentrated using a 1 kDa molecular weight cutoff membrane (MWCO). The pH was adjusted to neutral, and the fluid was sterile filtered and vialed in injection containers. Examples of r-aprotinin's behavior through the purification process are shown in Fig. 5.3.

As noted previously, many measures for recombinant product accumulation have been used in plant literature. For r-aprotinin, protein accumulation is reported as milligrams per kilogram of fresh weight of extracted tissues. This strategy normalizes many variables encountered in the extraction, purification, or formulation steps. Further, it provides a practical measure of crude production level from which to base predictable economics. GENEWARE® production of r-aprotinin in

greenhouse-grown *Nicotiana* plants showed crude and purified yields of ~750 mg/kg and 400 mg/kg, respectively. Field-produced plants showed crude and purified yields of ~300 mg/kg and 150 mg/kg, respectively. Field production was associated with a ~5-fold reduction in manufacturing costs compared with greenhouse production plants. Using either production method, these results suggest transient expression offers superior yields than transgenic approaches (Azzoni et al. 2002; Rival et al. 2008; Tissot et al. 2008; Zhong et al. 2007). The exploitation of agriculture scale allows production of 1 kg of purified r-aprotinin from 2500 sq. ft of greenhouse space or 1.5 acres of field-transfected *Nicotiana* plants (Pogue et al. 2010). These data demonstrate that transient plant production systems can provide product quantity and economies of scale that are more competitive than stably transformed plant systems for recombinant protein production.

5.3.3 r-Aprotinin Characterization

Standard release methods were used to characterize r-aprotinin and compare with the pharmaceutical standard (Pogue et al. 2010). Table 5.2 lists release tests and provides a comparison of results from greenhouse-produced r-aprotinin product with that of Trasylol®. In various tests for protein identity, including tryptic peptide analysis, amino acid analysis, and reactivity with anti-aprotinin mAb, both proteins provided identical results (Table 5.2; data not shown). Each protein showed a 6,512 Da mass as determined by MALDI mass spectrometry (Table 5.1). The potency of the proteins was also similar. Indeed, the r-aprotinin showed consistently higher kallikrein inactivation units (KIU) per mg of protein than Trasylol® (Table 5.1). Purity analyses showed no detectable protein impurities by overloaded SDS-PAGE, exact migration pattern on gels, similar reverse-phase high-pressure liquid chromatography (RP-HPLC; Fig. 5.3; Table 5.1), and immunoassays (data not shown). The GENEWARE® r-aprotinin product showed no immunoreactivity with a polyclonal antibody generated against crude *Nicotiana* protein extracts. These data provided additional support for the absence of host-derived proteinaceous impurities in the final product (data not shown). The stability of the liquid formulation of greenhouse-produced r-aprotinin at 4 °C was monitored over a 31-month period (Table 5.3). No significant changes in the purity, protein concentration, and specific activity were observed during the stability test (Table 5.3).

Comparison of greenhouse- and field-produced r-aprotinin did not reveal significant differences in the products (Table 5.3; Pogue et al. 2010). The potency of the field product was comparable with that of the greenhouse-produced API (Table 5.3). Purity achieved from field-grown materials and identity analyses revealed identical results (examples provided electrospray ionization time-of-flight mass spectrometry [ESI-TOF MS], appearance, and SDS-PAGE). The final bulk drugs were vialed under different conditions, but each showed concentrations that met predetermined bulk drug release specifications. These results demonstrate the consistency and quality of the GENEWARE® r-aprotinin produced under controlled greenhouse and

Table 5.1 Test methods and results of aprotinin product comparisons

Assay	Comparative attribute	8r-Aprotinin	Trasylol®
Identity by tryptic digest MALDI-TOF MS[a] mass mapping	Conforms with bovine lung aprotinin predicted tryptic fragments and fragment derivatives (84 % amino acid coverage)	Conforms	Conforms
Identity by MALDI-TOF MS	6,512 Da ± 0.05 %	6,512 Da	6,512 Da
Identity by amino acid analysis	Conforms with bovine lung aprotinin amino acid composition	Conforms	Conforms
Purity by SDS-PAGE[b]	Purity	>99 %	>99 %
Purity by RP-HPLC[c]	Purity	87.6 % + 12.4 (Ox)%	86.3 % + 5.7 (Ox)%
Purity by GC/MS[d] small molecular weight host toxicants	Purity	Comparable levels of target compounds	Comparable levels of target compounds
Purity by appearance	Clear, colorless, free of visible particles	Clear, colorless, particle free	Clear, colorless, particle free
Potency by specific activity[e]	>6,500 KIU/mg protein or >5.0 TIU/mg protein	7,175 KIU or 5.7 TIU	6,859 KIU or 5.4 TIU
Endotoxin[f]	<1 EU/28 mg	<1 EU/28 mg	<1 EU/28 mg

[a]Matrix-assisted laser desorption–ionization time-of-flight mass spectrometry (MALDI-TOF MS)
[b]Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)
[c]Reverse-phase high-pressure liquid chromatography (RP-HPLC) method separates non-oxidized and oxidized forms of r-aprotinin
[d]Gas chromatography (GC); mass spectrometry (MS)
[e]Kallikrein inactivation unit (KIU); trypsin inhibitory unit (TIU)
[f]Endotoxin units (EU)

field conditions (Table 5.3). The comparability to the product Trasylol® supports the use of transient plant expression to produce pharmaceutical candidates for drug development purposes.

5.4 Addressing Oxidation Concerns

5.4.1 Oxidation of Methionine Residue

The precision of the RP-HPLC methods was increased to study minor aprotinin variants present in each preparation. The Trasylol® product showed truncated aprotinin species, including lacking Ala58 and Gly57 (*des*Ala58 and *des*Ala58Gly57 species), and various oxidized aprotinin species present at 8 %

Table 5.2 Test methods and results of r-aprotinin comparisons (greenhouse vs. field grown)

Assay	Comparative attribute	r-Aprotinin (greenhouse)[a]	r-Aprotinin (field)[b]
Identity by ESI-TOF MS[c]	Average molecular mass between 6,508.2 and 6,514.8 Da	6,511.4 Da	6,511.8 Da
Purity by SDS-PAGE[d] (reduced)	≥95 % of r-aprotinin as determined by densitometry (% band)	>99 %	>99 %
Protein concentration by UV absorbance	≥5.0 mg/mL	21.3 mg/mL	18.3 mg/mL
Purity by appearance	Clear, colorless to amber, free of visible particles	Clear, light yellow, particle free	Clear, light yellow, particle free
Potency by TIU[e]	>5.0 (B) TIU/mg protein	5.7 TIU/mg	5.6 TIU/mg
Endotoxin[f]	<1 EU/28 mg	<1 EU/28 mg	<1 EU/28 mg

[a]Lot 07A0009
[b]Lot O8A0025
[c]Electrospray ionization time-of-flight mass spectrometry (ESI-TOF MS)
[d]Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)
[e]Trypsin inhibitory unit (TIU)
[f]Endotoxin units (EU)

Table 5.3 Stability testing at 4 °C of r-aprotinin product[a]

Assay (# months)	Purity[b] (%)	Protein concentration[c] (mg/mL)	Potency[d] (TIU/mg)
0	100	21.6	6.1
3	Not determined	21.5	5.7
6	Not determined	21.3	5.4
12	Not determined	21.0	6.4
16	Not determined	21.0	5.1
24	Not determined	21.3	5.5
31	>99	21.6	6.1

[a]Lot 07A0009
[b]Purity—sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and densitometry; release specification ≥95 %
[c]Concentration—optical density $(OD)_{280}$ and bicinchoninic acid method; release specification ≥5.0 mg/mL
[d]Trypsin inhibitory unit (TIU); release specification ≥5.0 TIU/mg

and 5.7 % of total product, respectively. No detectable truncated species were observed in the r-aprotinin product. However, it showed enhanced amounts of oxidized forms at 12.4 % of the final product (Table 5.1). Oxidation of methionine 52 of aprotinin is well known in the literature (Concetti et al. 1989). Indeed, oxidation of the methionine residue was noted in plastid-produced aprotinin in specific plant lines (Tissot et al. 2008). As found with native aprotinin, purification and testing of the potency of the oxidated species showed no significant difference from the non-oxidized fraction (data not shown; Concetti et al. 1989).

The oxidized form of the aprotinin product, although active, can lead to batch-to-batch variations which could impact product immunogenicity in humans or other pharmacokinetic or dynamic properties. The oxidized protein can be removed at the expense of protein yield by exploiting differences in physico-biochemical characteristics of the oxidized protein using a distinct reverse-phase chromatography method (RP-HPLC; Pogue et al. 2010). Ultimately, the RP-HPLC approach was used. However, manners to reduce oxidation were extensively evaluated.

5.4.2 Conditions Impacting Oxidation

The source of the oxidation and methods to control it during the production process were explored. Nb and *N. excelsiana* plants were tested for r-aprotinin oxidation status when grown under different light intensities and under different duration day/night cycles. These tests showed minimal impact of light intensity and day cycle length on the oxidation status of methionine 52. However, higher light intensities and longer day length produced much larger plants with much greater r-aprotinin yields on a per plant (or per/unit space) basis. In contrast, timing of plant harvest postinoculation did show significant impact on r-aprotinin oxidation. Oxidation increased proportionally from 10 to 18 days postinoculation. Comparison of older tissues versus younger leaf tissues showed higher levels of oxidation in older plant leaves (3.1 % in younger tissues compared with 5.7 % in older tissues). Finally, greater oxidation was observed in r-aprotinin obtained from field environments compared with controlled greenhouse conditions. Therefore, plant culture conditions can exert some impact on the oxidation status of r-aprotinin.

5.4.3 Aprotinin Variants Showing No Oxidation

To eliminate the oxidation issue altogether, genetic variants of r-aprotinin were constructed substituting Gln_{52}, Val_{52}, and Leu_{52} in place of Met_{52}, as well as deletion of this amino acid position altogether [(−) Met_{52}]. Aprotinin variants were subcloned into GENEWARE® vectors and expressed in *N. excelsiana* plants. Proteins were individually extracted, purified, and compared (Fig. 5.4). Similar product yields were noted in the Gln_{52} and Leu_{52} substitutions compared with wild-type r-aprotinin in both Nb and *N. excelsiana* plants. The Val_{52} substitution accumulated at 80 % of that of the wild-type protein, whereas the (−) Met_{52} variant accumulated at <30 % of the control. Gel migration and MALDI-TOF profiles showed expected sizes for each product (Fig. 5.4; Table 5.4) and near homogeneity of purification process as compared with Trasylol®. No oxidation was noted upon mass spectrometry or RP-HPLC analysis of any species (data not shown). The activities of the variants were identical to Trasylol® and wild-type r-aprotinin produced in plants using absolute and relative value calculations. The lone

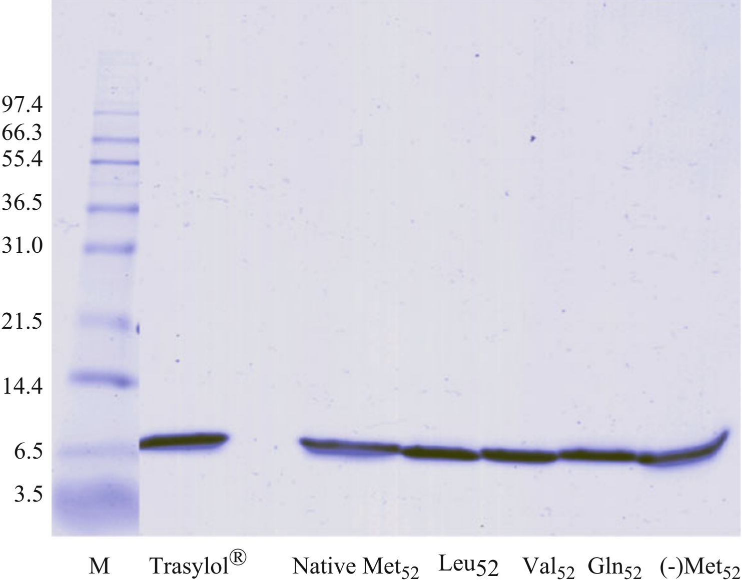

Fig. 5.4 SDS-PAGE analysis of Trasylol® and purified r-aprotinin and variants. Native r-aprotinin (Met_{52}) and variants: Leu_{52}, Val_{52}, Gln_{52}, and (−) Met_{52} are noted as lane definitions. Recombinant aprotinin variants were purified to virtual homogeneity from transfected *Nicotiana excelsiana* plants and compared using SDS-PAGE with each other and Trasylol®. Lane markers indicate the identity of the loaded protein. Molecular weight markers containing known molecular weight proteins are loaded at *left*. Proteins were analyzed using 4–12 % Bis-Tris sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and subjected to Coomassie Brilliant Blue staining

Table 5.4 Characteristics of r-aprotinin non-oxidized variants

Aprotinin	Mass (D)	KIU/mg	EPU/mg kcat/mg	IU_{BAPA}/mg	Relative IU[a]
Trasylol®	6,511.83	7,116	3.95	11.57	100
r-Aprotinin native Met_{52}	6,512.29	7,196	4.00	11.70	100
r-Aprotinin Gln_{52}	6,494.28	7,147	3.97	11.62	100
r-Aprotinin Leu_{52}	6,510.74	7,218	4.01	11.74	100
r-Aprotinin Val_{52}	6,479.76	7,161	3.98	11.64	100
r-Aprotinin (−) Met_{52}	6,381.93	6,847	3.8	11.13	96

[a]Relative IU is expressed as inhibition activity of r-aprotinin relative to the IU expected for pure bovine aprotinin (Fritz and Wunderer 1983)

exception is the (−) Met_{52} variant that showed slightly lower specific activity (Table 5.4). Therefore, protein oxidation can be controlled while maintaining high activity by modifications and optimization of plant growth conditions, addition of additional purification steps, or by genetic modification of the product. The rapid nature of GENEWARE® testing of variants makes the testing of such genetic

variants rapid and a viable option when subtle differences in protein composition can be tolerated in a final product.

5.5 Conclusions

Recombinant aprotinin can be readily produced using transient expression methods in a cost-competitive and pharmaceutically acceptable fashion from transfected *Nicotiana* plants. Production under controlled greenhouse conditions and in relatively noncontrolled field conditions results in products that show remarkable consistency, quality, and stability (Tables 5.1, 5.2, 5.3, and 5.4). Although plant-produced aprotinin is currently not approved for human use, its integration in cell culture as a proteinase inhibitor is common. The r-aprotinin produced from *Nicotiana* plants has been marketed in collaboration with Sigma Aldrich since 2005 and has been widely used in the industry for a variety of functions. The high purity and quality of product allows its integration into research and development as well as cGMP manufacturing processes as a fermentation or purification excipient (Pogue et al. 2010). As regulators continue to evaluate the safety and efficacy of aprotinin as a pharmaceutical product, plant production stands ready to provide the active pharmaceutical ingredient as needed. These human applications were the motivation of the initial production of r-aprotinin, and we hope that this goal will be eventually realized.

It should be noted that the rapid nature of the GENEWARE® allowed the generation and testing of r-aprotinin variants that show equivalency in activity, yet lack oxidation issues that plague both bovine- and plant-derived products (Fig. 5.4; Table 5.4). This speed to product, producing milligrams of product in as little as two weeks and production of grams in a month or more, is dramatically shorter than the requirements to transfect, select, establish, and characterize mammalian cells, transgenic animal, or traditional transgenic plant-based systems. Indeed, the yields that can be expected from these systems can be quite high, ranging from 0.3 to 0.75 g/kg when extracting >100 kg of crude plant material, and are >10-fold greater than production levels of the same proteins in transgenic plant systems (Pogue et al. 2010).

The r-aprotinin variants may have future pharmaceutical interest. Since aprotinin is a xenographic protein, the substitution of a single amino acid would not be expected to enhance the immunogenicity of the product in humans over the current bovine-derived product. Indeed, aprotinin was suggested for single use in humans due to potential immunological response upon re-administration. However, the properties of the Met_{52} substitution variants will need to be explored in human systems before definitive conclusions can be drawn.

5.6 Future Directions

Plants have been discussed as a cost-effective alternative for pharmaceutical protein production to the current mammalian or microbial cell-based systems due to cost and safety advantages (Floss et al. 2007; Lico et al. 2008; Ma et al. 2003; Plasson et al. 2009). Recombinant aprotinin provides a fine case study to demonstrate that plants can deliver on this promise. The quality of the product and its production on a large and relevant scale has been demonstrated, and release criteria match those for analogous product approved for pharmaceutical use by the FDA. More opportunities exist for additional pharmaceutical products to be produced by transient methods in plants. Detailed review of the GENEWARE® systems provides further demonstration that quality biologics can be produced using nonfood/non-feed, non-genetically modified plants (reviewed in Pogue et al. 2002, 2010; Pogue and Holzberg 2013). Transient expression strategies offer the speed to tailor recombinant products to the biochemical challenges (such as oxidation) or clinical needs. Demonstration of the quality of plant-derived proteins in nonclinical applications, such as cell culture excipients and other applications, where high-quality products are required will increase confidence of the pharmaceutical industry to accept and test plant systems as sources for recombinant products. Continued sales and use of r-aprotinin is such an "ambassador" product. The data provided for r-aprotinin provided here strongly contend that transient plant expression systems offer a legitimate cost-competitive alternative for recombinant protein production.

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References

Azzoni AR, Kusnadi AR, Miranda EA, Nikolov ZL (2002) Recombinant aprotinin produced in transgenic corn seed: extraction and purification studies. Biotechnol Bioeng 80:268–276

Beierlein W, Scheule AM, Dietrich W, Ziemer G (2005) Forty years of clinical aprotinin use: a review of 124 hypersensitivity reactions. Ann Thorac Surg 79:741–748

Concetti A, Angeletti M, Fioretti E, Ascoli F (1989) Selective oxidation of methionine residues in Kunitz-type protease inhibitors. Biol Chem Hoppe Seyler 370:723–728

Dias DA, Urban S, Roessner U (2012) A historical overview of natural products in drug discovery. Metabolites 2:303–336

Farnsworth NR, Akerele O, Bingel AS, Soejarto DD, Guo Z (1985) Medicinal plants in therapy. Bull World Health Organ 63:965–981

Fischer R, Twyman RM, Schillberg S (2003) Production of antibodies in plants and their use for global health. Vaccine 21:820–825

Fitzmaurice WP (2002) Interspecific Nicotiana hybrids and their progeny. US Patent no. 6,344,597

Floss DM, Falkenburg D, Conrad U (2007) Production of vaccines and therapeutic antibodies for veterinary applications in transgenic plants: an overview. Transgenic Res 16:315–332

Fritz H, Wunderer G (1983) Biochemistry and applications of aprotinin, the Kallikrein inhibitor from bovine organs. Arzneimittelforschung 33:479–494

Hiatt A, Cafferkey R, Bowdish K (1989) Production of antibodies in transgenic plants. Nature 342:76–78

Laskowski M Jr, Kato I (1980) Protein inhibitors of proteinases. Annu Rev Biochem 49:593–626

Lico C, Chen Q, Santi L (2008) Viral vectors for production of recombinant proteins in plants. J Cell Physiol 216:366–377

Ma JKC, Drake PMW, Christou P (2003) The production of recombinant pharmaceutical proteins in plants. Nat Rev Genet 4:794–805

Maffulli N, Walley G, Sayana MK, Longo UG, Denaro V (2008) Eccentric calf muscle training in athletic patients with Achilles tendinopathy. Disabil Rehabil 30:1677–1684

Mangano DT, Tudor IC, Dietzel C (2006) The risk associated with aprotinin in cardiac surgery. NEJM 354:353–365

Mangano DT, Miao Y, Vuylsteke A, Tudor IC, Juneja R, Filipescu D, Hoeft A, Fontes ML, Hillel Z, Ott E, Titov T, Dietzel C, Levin J (2007) Mortality associated with aprotinin during 5 years following coronary artery bypass graft surgery. JAMA 297:471–479

Maxmen A (2012) Drug-making plant blooms. Nature 485:160

Munoz JJ, Birkmeyer NJO, Birkmeyer JD, O'Conner GT, Dacy KJ (1999) Is epsilon-aminocaproic acid as effective as aprotinin in reducing bleeding with cardiac surgery? A meta-analysis. Circulation 99:81–89

Newman DJ, Cragg GM (2007) Natural products as sources of new drugs over the last 25 years. J Nat Prod 70:461–477

Nordic Pharma Group (2012) http://www.nordicpharmagroup.com/art-4-4-38-the-nordic-group-acquires-rights-to-trasylol-from-bayer-healthcare.html. Cited 09.08.2013

Odum JN (2001) Biotech manufacturing: is the crisis real? Pharm Eng 21:22–33

Orchard J, Massey A, Brown R, Cardon-Dunbar A, Hofmann J (2008) Successful management of tendinopathy with injections of the MMP-inhibitor aprotinin. Clin Orthop Relat Res 466:1625–1632

Plasson C, Michel R, Lienard D, Saint-Jore-Dupas C, Sourrouille C, de March GG, Gomord V (2009) Production of recombinant proteins in suspension-cultured plant cells. Methods Mol Biol 483:145–161

Plesha MA, Huang TK, Dandekar AM, Falk B, McDonald K (2009) Optimization of the bioprocessing conditions for scale-up of transient production of a heterologous protein in plants using a chemically inducible viral amplicon expression system. Biotechnol Prog 25:722–734

Pogue GP, Holzberg S (2013) Transient virus expression systems for recombinant protein production in dicot- and monocotyledonous plants. In: Dhal NK, Sahu SC (eds) Plant science. InTech Press, Rijeka, Croatia, pp 189-216. ISBN 980-953-307-033-4

Pogue GP, Lindbo JA, Dawson WO, Turpen TH (1998) Tobamovirus transient expression vectors: tools for plant biology and high-level expression of foreign proteins in plants. In: Gelvin SB, Schilperoot RA (eds) Plant molecular biology manual, vol L4. Kluwer Academic, Dordrecht, pp 1–27

Pogue GP, Lindbo JA, Garger SJ, Fitzmaurice WP (2002) Making an ally from an enemy: plant virology and the new agriculture. Annu Rev Phytopathol 40:45–74

Pogue GP, Vojdani F, Palmer KE, Hiatt E, Hume S, Phelps J, Long L, Bohorova N, Kim D, Pauly M, Velasco J, Whaley K, Zeitlin L, Garger SJ, White E, Bai Y, Haydon H, Bratcher B (2010) Production of pharmaceutical-grade recombinant aprotinin and a monoclonal antibody product using plant-based transient expression systems. Plant Biotechnol J 8:638–654

Rademakers LM, Gründeman PF, Bolderman RW, van der Veen FH, Maessen JG (2009) Stability of an autologous platelet clot in the pericardial sac: an experimental and clinical study. J Thorac Cardiovasc Surg 137:1190–1194

Rival S, Wisniewski JP, Langlais A, Kaplan H, Freyssinet G, Vancanneyt G, Vunsh R, Perl A, Edelman M (2008) Spirodela (duckweed) as an alternative production system for pharmaceuticals: a case study, aprotinin. Transgenic Res 4:503–513

Sedrakyan A, Treasure T, Elefteriades JA (2004) Effect of aprotinin on clinical outcomes in coronary artery bypass graft surgery: a systematic review and meta-analysis of randomized clinical trials. J Thorac Cardiovasc Surg 128:442–448

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

Shivprasad S, Pogue GP, Lewandowski DJ, Hidalgo J, Donson J, Grill LK, Dawson WO (1999) Heterologous sequences greatly affect foreign gene expression in tobacco mosaic virus-based vectors. Virology 255:312–323

Sourrouille C, Marshall B, Liénard D, Faye L (2009) From Neanderthal to nanobiotech: from plant potions to pharming with plant factories. Methods Mol Biol 483:1–23

Stamou SC, Reames MK, Skipper E, Stiegel RM, Nussbaum M, Geller R, Robicsek F, Lobdell KW (2009) Aprotinin in cardiac surgery patients: is the risk worth the benefit? Eur J Cardiothorac Surg 36:869–875

Taylor L (1996) The healing power of rainforest herbs. Square One Publishers, City Park, NY

Thiel KA (2004) Biomanufacturing, from bust to boom....to bubble? Nat Biotechnol 22:1365–1372

Tissot G, Canard H, Nadai M, Martone A, Botterman J, Dubald M (2008) Translocation of aprotinin, a therapeutic protease inhibitor, into the thylakoid lumen of genetically engineered tobacco chloroplasts. Plant Biotechnol J 6:309–320

Zhong Q, Xu L, Zhang C, Glatz CE (2007) Purification of recombinant aprotinin from transgenic corn germ fraction using ion exchange and hydrophobic interaction chromatography. Appl Microbiol Biotechnol 76:607–613

Part II
Vaccines

Chapter 6
Influenza Virus-Like Particles Produced in *Nicotiana benthamiana* Protect Against a Lethal Viral Challenge in Mice

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6.1 Introduction

In 1997–1998, when the world first learned about a deadly avian influenza strain in Hong Kong, none of the existing commercial approaches to vaccine manufacturing were ready to respond to what was feared might become a global health catastrophe. Although the probability of a new pandemic had been increasing steadily, and warnings had come repeatedly from the scientific community, little effort had been made to prepare the world to respond to such a pandemic prior to 1997–1998. In the view of the vaccine manufacturers, the existing surge capacity was either considered to be adequate, or it was considered that the expansion of such capacity was too high-risk a commercial opportunity to justify significant investment. In brief, we (in resource-rich settings at least) thought that we were OK. Supply contracts were put in place in some of the wealthier countries as a safety net, although it was well understood that, in the case of a rapidly moving serious pandemic, no one could expect a substantial number of vaccine doses before 6–8 months, i.e., potentially long after the first wave of infection.

We were lucky in 1997–1998 and have remained lucky since that time. The H5 avian strains, although extremely pathogenic, have been inefficient at spreading from birds to humans and have not spread among humans. Sporadic episodes of limited transmission from birds have occurred since 1997–1998, mostly in Asia, but no sustained human-to-human transmission has occurred. However, the alertness among the scientific community remains high as these avian strains are actively mutating and are only a few mutations away from becoming able to spread to and among humans.

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Despite the grumbling concern about avian viruses (recently revived with the emergence of highly pathogenic H7N9 strains in China in 2013), it was the 2009–2010 swine H1N1 influenza pandemic (pH1N1) that revealed the true limitations of the existing influenza vaccine manufacturing technologies. On May 26, 2009, the World Health Organization (WHO) recommended rapid development of vaccines to address the pH1N1 pandemic, and the first reassortant viruses were made available on May 27, 2009 (WHO 2009). Although Wyman and colleagues had earlier predicted that egg-based manufacturing would be able to supply at least 60 million vaccine doses within 5 months of the declaration of a pandemic (Wyman 2007), the actual vaccine output in the 2009–2010 pH1N1 pandemic was much lower. In fact, only three million doses of live attenuated vaccine were available by October 2009. The first doses for split vaccine became available shortly thereafter but in numbers far below expectations. Fortunately, the pH1N1 strain had a low mortality rate compared to the 1918–1919 pandemic strain. Had the pH1N1 pandemic been more severe, the global human cost of the delays in vaccine production could have been catastrophic.

This is not to say that the vaccine industry remained idle and did not take the threat of pandemic seriously. In the early 2000s, when it seemed that the threat of avian influenza pandemic was here to stay, a number of companies had started looking at other approaches to pandemic vaccine manufacturing or at vaccines that would be more cross-reactive (i.e., for pre-pandemic or prophylactic use). They also began to evaluate vaccines that could be manufactured more rapidly and that would require a lower dose (i.e., antigen sparing) or that would be effective with a single dose. Since humans are generally immunologically naive to emerging pandemic strains, it was widely believed that the one-dose non-adjuvanted immunization approach taken for seasonal influenza would not be as effective for pandemic influenza. This expectation has largely been borne out in clinical trials with H5 vaccine candidates but proved not to be the case with the pH1N1 pandemic.

In 2007, Medicago Inc. decided to invest in the development of candidate pandemic vaccines. At that time, the vaccine market was rapidly expanding with an increasing number of countries seeking to establish local manufacturing capacity, with national health organizations pushing for increased vaccination programs and exciting developments for many new vaccine applications, i.e., unmet medical needs. As outlined above, these pressures were mounting despite little or no improvement in vaccine manufacturing technologies. This combination of events represented an important opportunity for Medicago.

Our objective was to develop a manufacturing approach that would offer what was desperately needed in a pandemic vaccine platform: surge capacity, speed, adaptability, and affordable cost per dose. As we were developing a recombinant platform and as this would provide us with the ability to modulate the composition of our antigen components, one of our goals was also to differentiate ourselves from existing pandemic vaccines with a product with greater cross-reactivity and efficacy.

We saw several advantages in moving into the pandemic field; it was an industrial area where the existing technologies were not meeting the expectations

of the national and global health agencies. Our perception was that the egg-based vaccine manufacturing platform was well established but had evolved only slowly due to modest market opportunities for vaccines in the last decades. In addition, the use of eggs as raw material for vaccine production was increasingly challenged from a sustainability perspective, especially for emerging economies. Thus, the technology we would be confronting was already being challenged by its end users for its sustainability, its limited surge capacity, and more recently for its adaptability (e.g., the first isolates of H5N1 killed the eggs).

6.2 Development of an Influenza Vaccine

6.2.1 The Design of Medicago's Influenza Vaccine

There were a limited number of reports on the production of influenza proteins in plants prior to our own attempts, only some of which had been tested for antigenicity. Evidence of such production had just been disclosed in 2004 through a patent application by Cardineau et al. (2004). He and his colleagues had demonstrated that the hemagglutinin (HA) protein of an influenza A virus (A/turkey/Wisconsin/1968 (H5N9)) accumulated in calluses of *Nicotiana tabacum*. When used for immunization, this partially purified protein induced an immune response in mice in the presence of complete Freund's adjuvant. Although this represented an interesting proof of principle, the use of Freund's adjuvant meant that this vaccine could not be used in humans. Therefore, the question remained how an HA antigen produced in plants would compare to HA antigens produced by other recombinant platforms or to HA in virions produced in eggs. However, most of the prior attempts to make influenza antigens in plants had focused primarily on soluble forms of these proteins.

Influenza is an enveloped virus that assembles by budding from the host cell plasma membrane. As a result, the influenza virus envelope is made of a lipid bilayer of host cell origin. In its natural hosts, budding occurs at specialized regions of the plasma membrane called lipid rafts. Its major surface components consist of glycoprotein trimers (hemagglutinin) or tetramers (neuraminidase). Each of these glycoproteins has a globular domain attached to a stalk or stem domain that protrudes from the lipid bilayer envelope to which they are anchored by a short transmembrane domain. Both hemagglutinin and neuraminidase are synthesized and matured in the ER and are glycosylated. In 2007, it was not clear if N-glycan structure played an important role in the antigenicity for either of these surface proteins and, thus, if glycosylation would be a challenge for making influenza antigens in plants.

Given that each plant cell is surrounded by a cell wall composed of a tight matrix of cellulose microfibrils and pectin, with little porosity, any virus (or viruslike particle (VLP)) budding from the plant plasma membrane would effectively be

"trapped" within that matrix. Although this route of egress is common for many viruses that infect non-plants, budding from the plasma membrane is not an evolutionary track that plant viruses have followed. While there have been numerous reports showing that capsid proteins of mammalian viruses can spontaneously assemble in plant cells (D'Aoust et al. 2010), it was initially a concern of Medicago's influenza project that budding of influenza surface proteins as VLPs from the plasma membrane of plant cells would be restrictive. It was feared that these proteins would mostly accumulate as immature, ER-bound proteins. As several reports had shown that soluble or membrane-bound but non-assembled influenza proteins had lower antigenicity than highly structured particles and in spite of the challenges ahead, our objective remained to produce fully assembled virus-like particles (VLPs).

At that time, several groups had shown that influenza surface glycoproteins could assemble into true VLPs when produced in mammalian cells or insect cells in culture. However, by 2007, there had been only one report suggesting that plant cells could produce enveloped VLPs (Mason et al. 1992). In that report, small particles (22–35 nm) resembling HepB noninfectious virions had been isolated from transgenic tobacco plants expressing the HepB major surface protein. However, no structural characterization of the particles had been performed, and the nature of the envelope was unknown.

Hemagglutinin (HA), the most abundant surface protein of influenza viruses, has long been considered the major antigenic determinant for this virus. Indeed, by 2007, there had been many reports showing that HA by itself, either from split viruses or recombinant sources, was able to trigger the production of neutralizing and protective antibodies. Neuraminidase (NA), the other major influenza surface glycoprotein, was also known to be immunogenic but was less attractive as a vaccine antigen since anti-NA IgGs did not prevent infection. In light of these observations, it was decided that HA would be our first antigen target. It was not known if other viral proteins (or helper proteins) would be required to produce a true influenza VLP in plants as the available prior art (Gómez-Puertas et al. 2000) suggested that the M1 protein was required for proper assembly and budding of influenza VLPs. Although many strains of pandemic avian H5N1 influenza had been characterized since 1997, the most prominent strain in circulation at that time was the A/Indonesia/5/05 strain and we decided to develop our first candidate vaccine with the HA from that wild-type strain.

6.2.2 Production of the First Plant-Made Influenza Vaccine Candidate

The H5 hemagglutinin from the Indonesia strain lent itself well to transient expression in *Nicotiana benthamiana*. We have previously described the approach (D'Aoust et al. 2008) to co-express HA with the P1/HcPro inhibitor of silencing

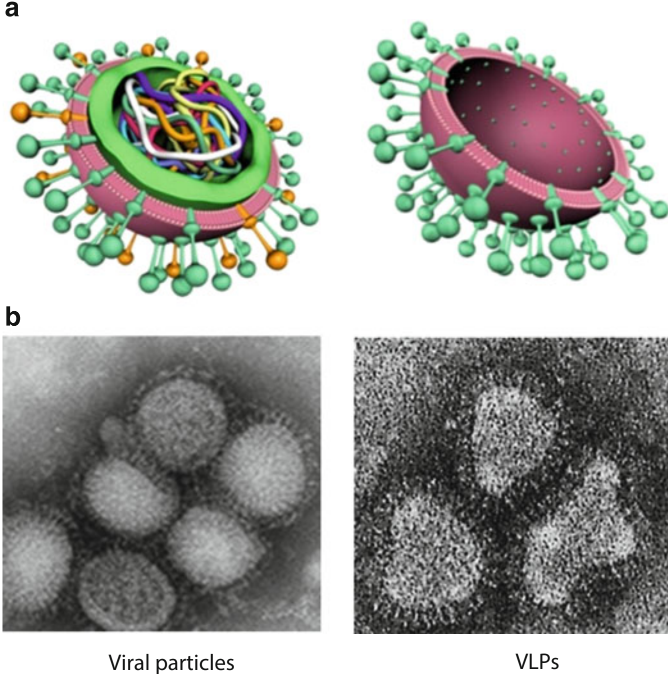

Fig. 6.1 Schematic representation of the structural characteristics of viral particles and plant-made VLPs. (**a**) Cross-section showing structural and composition differences of viral particles (*left*) and plant-made VLPs. (**b**) Transmission electron microscopy images of influenza viruses and plant-made VLPs

from the potato virus Y. For this approach, the HA gene was placed under the control of the alfalfa plastocyanin promoter and terminator in a first construct and the HcPro gene under the CaMV promoter in a second construct. Each construct was transferred to *Agrobacterium tumefaciens*, and single *A. tumefaciens* isolates were chosen for the preparation and characterization of master and working cell banks. Transient expression occurred when the two *A. tumefaciens* inocula were mixed and vacuum infiltrated into *N. benthamiana* leaves (D'Aoust et al. 2008).

Expression of the H5 HA gene in *N. benthamiana* led to several surprising observations. While attempting to concentrate and purify HA from whole extracts, it became obvious that the viral protein was predominantly located in structures far larger than the expected trimers. The combination of differential centrifugation, size exclusion chromatography, and light scattering strongly suggested that the HA was assembled in large macromolecular structures (Fig. 6.1). These were later identified as VLPs by transmission electron microscopy. As described in D'Aoust (2008), two sets of observations brought additional confirmation that the HA alone had efficiently assembled into VLPs in *N. benthamiana*. First, the detailed biochemical analysis of the purified particles indicated that the envelope in which the HA was embedded was made of phospholipids with a high relative abundance of

Table 6.1 Lipids identified by LC/MS/MS in pilot plant engineering lots as of June 8, 2010, that are characteristic of lipid rafts

Phosphatidylethanolamine (PE) 16:0, 18:2	Glucosylceramide d18:2, h16:0
Phosphatidylcholine (PC) 16:0, 18:2	Ceramide t18:0, c22:0
Phosphatidylserine (PS) 16:0, 18:2	Hydroxy-ceramide t18:1, h22:0
Cholesterol	B-sitosterol
Stigmasterol	Campesterol

lipids characteristic of lipid rafts (Table 6.1). Second, EM images of transfected plant leaf tissue clearly indicated that VLPs were concentrating at a small number of budding sites on the cell periphery, where the plasma membrane had retracted from the cell wall to create invaginations or pouches (D'Aoust et al. 2008). This was an exciting moment as it was the first demonstration that VLPs would form through the action of budding from a plant cell plasma membrane and that HA alone was able to drive VLP budding. Furthermore, the particles appeared to be budding at lipid rafts, as in their natural host cells.

In the end, purifying the VLPs in preparation for preclinical and clinical studies proved to be more of a challenge than expression of the HA antigen. As described in Landry et al. (2010), VLPs were first purified from 20 kg lots of infiltrated *N. benthamiana* leaves by a process that used mechanical extraction to release the VLPs. As HA has the ability to stick to other proteins, protein bodies, and other polymeric structures, using mechanical extraction implied that we used specific conditions to prevent the VLPs from interacting with cell debris and host cell polymers during the first steps of primary recovery. Elimination of debris and major host cell components required the use of controlled coagulation under mild heat and acidic conditions (42 °C, pH 5.3) coupled with filtration through diatomaceous earth.

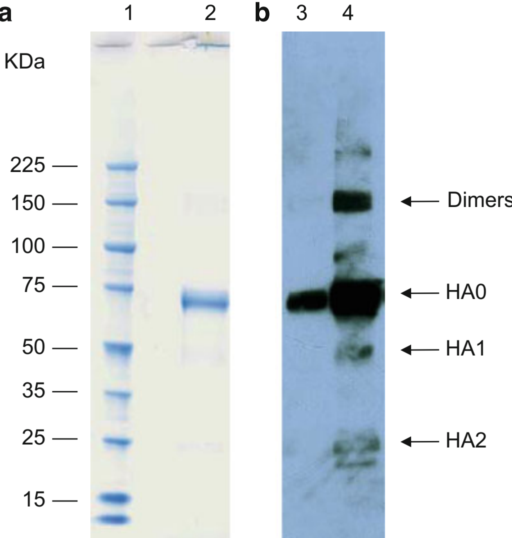

Fig. 6.2 HA identity and purity of GMP lots. (**a**) Coomassie-stained SDS-PAGE analysis (*lane 1*). Molecular weight markers (*lane 2*) 2.5 μg of H5 VLP Influenza vaccine in reducing conditions. (**b**) Western blot analysis. (*Lane 3*) 50 ng and (*lane 4*) 500 ng of the H5 VLP Influenza vaccine. Polyclonal antibodies raised against H5N1 (strain A/Indonesia/5/05) were used as primary antibody for Western blot analysis. HA0, complete hemagglutinin protein consisting of domains HA1 and HA2; HA1, domain 1 of hemagglutinin protein; HA2, domain 2 of hemagglutinin protein

VLPs were concentrated from clarified extracts by tangential flow filtration (TFF). Preserving the ultrastructure of the VLPs, and preventing them from aggregation, was ensured by the combined use of salt and detergent. Passage on a Poros HQ column (pH 7.5) in a flow-through mode was used to remove endotoxins and contaminating DNA. The flow-through of the Poros HQ was loaded onto a Poros HS where VLPs were purified in a bind-elute mode. The VLPs were captured on an affinity Poros EP 250 coupled to bovine fetuin (Landry et al. 2010). VLPs were eluted from the fetuin affinity column under high salt and submitted to a second TFF step where they were brought to their final concentration in the desired formulation. This active ingredient (AI) was fully characterized, was shown to be of high purity and integrity (Coomassie-stained SDS gels; see Fig. 6.2), high potency (SRID assay), and was deemed of adequate quality (DNA and endotoxin content) for preclinical and clinical testing (Landry et al. 2010). The whole procedure, including seed production, biomass production, master and working *A. tumefaciens* cell bank production, inoculum production, infiltration, incubation, primary recovery, purification, and in-process testing, was eventually brought to operation under cGMP so that preclinical and clinical testing could be initiated. As a precautionary measure, an extensive product characterization program was also developed which allowed us not only to provide the information required for our regulatory filings (purity, integrity, DNA, and endotoxin) but also allowed us to learn more about the trace contaminants found in the preparations. The purification

procedures gave yields at the low end of our predetermined acceptable range at that stage, and it was understood that we would look for improvements of the process in order to increase the overall process yields prior to moving to larger clinical trials.

As this vaccine was, from a physicochemical perspective, a suspension of lipid bilayer nanoparticles covered with spikes of trimeric HA, stability in solution became a challenge when we reached high concentrations at the end of the purification sequence. A formulation had to be developed that would prevent aggregation between particles at high concentration at the time of preparation and during storage. A final formulation was developed that allowed the product (active ingredient and final formulation in vials) to remain stable as a monodisperse suspension of particles.

6.2.3 Preclinical Testing of the Plant-Made Influenza VLP Vaccine

Preclinical testing of our candidate H5-VLP vaccine started with immunogenicity studies in mice. In the first, exploratory dose-ranging study, two doses of our VLP vaccine were administered to mice with or without adjuvant (Alhydrogel®; (Cedarlane Laboratory, Burlington, Ontario, Canada)) and compared with two doses of a recombinant H5. The detailed description of this study appears in D'Aoust et al. (2008). This first experiment showed that the VLPs induced a much stronger hemagglutinin inhibition (HI) antibody response than the non-assembled H5. It further showed that two doses of as little as 0.1 μg induced mean HI titers well above 1:40. As there was an embargo on the use of the Indonesian H5N1 isolates (clade 2) for vaccine testing at that time, it was impossible to use the homologous strain for lethal challenges, and we had to rely on the use of heterologous strains. Thus, in follow-up mouse studies, we were able to show that doses of as low as 0.5 μg protected mice against a lethal challenge with one LD_{50} of the clade 1 isolate A/Vietnam/1194/04 (H5N1) and that doses of as low as 1 μg protected against ten LD_{50} of the clade 2 strain, A/Turkey/582/06.

The vaccine was then tested in ferrets, which are the most reliable small animal model for influenza. When ferrets are infected by influenza viruses, they develop symptoms similar to those seen in humans, and ferrets are sensitive to infection by the same strains as humans. As described in D'Aoust et al. (2010), our first ferret study assessed antibody reactivity to homologous and heterologous HA targets. It demonstrated that a single dose of 5 μg (adjuvanted) or two doses of 1 μg (adjuvanted) triggered a strong immune response that was considered to be protective according to the CHMP criteria against the homologous A/Indonesia/5/05 strain (see Table 6.2). Although these are criteria established to assess human responses, they are often used to interpret data from ferrets. This study also demonstrated that 2 alum-adjuvanted doses of our VLP vaccine triggered potent cross-reactive antibodies to heterologous strains of the same clade (A/Turkey/Turkey/1/05; A/Anhui/1/05) and a different clade (A/Vietnam/1194/04).

Table 6.2 Evaluation of the immune response of ferrets after immunization with H5-VLPs

	Response	Study group	
		1 μg	5 μg
1st injection	Percentage of 4-fold increase in HI titer >40 %	100 %	100 %
	Mean geometric increase >2.5	7.6	15.6
	Percentage of HI titer above 1/40 >70 %	60 %	100 %
2nd injection	Percentage of 4-fold increase in HI titer >40 %	100 %	100 %
	Mean geometric increase >2.5	82	93
	Percentage of HI titer above 1/40 >70 %	100 %	100 %

The vaccine was next tested in a challenge study in ferrets. Male Fitch ferrets were vaccinated with either two doses of our VLP vaccine (0.7, 1.8, 3.7, or 11 μg + Alhydrogel®) or placebo (PBS+ Alhydrogel®). Forty-five days after the boost injection, each group was challenged intranasally with a lethal dose (10× Ferret Lethal Dose) of A/Vietnam/1203/04. Animals were monitored for symptoms of illness throughout the challenge, and some animals were sacrificed during the challenge for analyses of viral load in nasal turbinates and lungs. The results of this study are described in details in Landry et al. (2010). The VLP vaccine triggered a significant dose-dependent HI response against the homologous strain and a more moderate response to heterologous strains of the same clade or of a different clade. Not surprisingly, the responses were stronger after the booster injections. However, the most striking result of this study was that the VLP vaccine provided complete cross-clade protection. In the first days of challenge, all ferrets from the placebo group developed severe symptoms of infection, and several of them were euthanized due to loss of >20 % of body weight. All vaccinated ferrets survived the challenge with only slight symptoms recorded. Viruses were found in the lungs and turbinates of all of the control ferrets, but otherwise only in the animals that had been vaccinated with the lowest doses.

6.2.4 Phase I Clinical Trial of the Plant-Made Influenza VLP Vaccine

Prior to filing our regulatory dossier to get authorization for a Phase I clinical trial, we brought one significant change to the expression system that had been used during early product development. The plastocyanin promoter and terminator that drove expression of the HA gene were replaced by the CPMV-HT system developed at the John Innes Institute (D'Aoust et al. 2010). This change helped to increase accumulation rates of the fully assembled VLPs and allowed us to start with a raw material that had higher concentrations of VLPs (μg VLPs per g fresh biomass).

The Phase I clinical trial was performed at the Vaccine Study Center of the McGill University Health Research Center. It was a randomized, double-blind, placebo-controlled trial designed to assess the safety and immunogenicity with approval from the Canadian Biologic and Genetic Therapies Directorate (BGTD) as an investigational new drug (CTA). This first study involved 48 healthy adult subjects.

The study design and procedures are described in detail in Landry et al. (2010). The principal exclusion criteria were not different from studies for comparable products entering Phase I, with the exception of known history of allergies to constituents of the H5-VLP vaccine or allergies to tobacco. Subjects with declared mild to moderate allergies to plant constituents (i.e., hay fever, allergies to ragweed) were not excluded from enrollment. Starting at the lowest dose (5 μg per dose), groups of subjects were randomized to receive either the vaccine or the placebo, and a review of safety data was performed by an independent panel after each wave of immunization. The study was completed in three waves at increasing dose levels. Subjects were observed for at least 2 h after each immunization for any symptoms of local or systemic reaction. Serum was collected before and 21 days after each immunization for immunological, biochemical, and hematological analyses.

The immune response to H5-VLP vaccination was assessed using three standard serologic assays: HI, microneutralization (MN), and single radial hemolysis (SRH). The HI assay estimates serum immunoglobulin titers that can prevent agglutination of HA-coated erythrocytes. Microneutralization is largely self-explanatory and measures the capacity of antibodies to block infection of susceptible cells. Although MN is a more functional measure of antibody response and is generally more sensitive than HI testing, there are no clear correlates between MN values and protection in humans. The SRH assay measures complement-mediated hemolysis as antigen–antibody immune complexes form in vitro. When a vaccine containing multiple viral proteins is tested, SRH provides an estimate of the response to all of the antigenic determinants in the vaccine and thus can overestimate the humoral response to the surface glycoproteins (HA and NA). Only the HI and SRH assay results are used by regulators in different jurisdictions in considering licensure of a product. It is important to note that all of these assays can be strongly influenced by the source of the virus or viral antigens used in testing. Until recently, all commercial vaccines were based on either egg- or tissue culture-adapted virus strains, and the "standard" or "reference" reagents for serologic testing were also derived from the same sources. Since the process of adaptation for optimal growth in eggs or tissue culture inevitably introduces mutations in these strains, the use of such "standard" reagents to evaluate vaccines based upon wild-type (WT) sequence information has the potential to introduce a systematic bias against newer vaccines. We have recently demonstrated that this potential for bias is indeed realized when "standard" reagents are used to evaluate responses to our VLP vaccines. We and others with candidate vaccines based on WT sequence data are now rapidly shifting to testing using WT reagents.

As this Phase I study was the very first experience with parenteral administration of a glycosylated VLP of plant origin, the humoral response to plant-specific glycans was also of considerable interest. Both IgG and IgE responses to different plant glycan motifs were measured by ELISA using either corn avidin (vs. egg avidin) or bromelain as the target. As noted above, most subjects with allergic

Table 6.3 Percent adverse events per group by treatment

	First dose				Second dose			
Adverse event	5 μg	10 μg	20 μg	Placebo	5 μg	10 μg	20 μg	Placebo
Local reaction								
Redness	2	4	9	3	8	2	5	5
Swelling	1	0	4	2	2	5	1	3
Pain	11	8	8	7	9	9	8	6
Systemic reactions								
Fever	0	0	0	0	0	0	0	0
Headache	4	6	2	4	6	5	1	2
Joint ache	0	1	1	0	0	2	0	1
Fatigue	0	4	1	1	4	3	2	2
Muscle ache	2	5	2	3	1	3	0	0
Feeling of general discomfort	2	2	0	1	2	2	0	0
Chills	0	2	0	1	1	1	0	0

Data are percentages. Adverse events up to 7 days after vaccination are reported

histories, including those with known allergies to plant materials (e.g., seasonal allergies, ragweed), were not excluded from the study.

6.2.5 Safety and Reactogenicity

The vaccine was well tolerated. There were no severe adverse effects (SAE) recorded either during the vaccination period, 21 days after the second injection, or during the 6-month follow-up period of the study. Pain at injection site, redness, and headaches were the most commonly reported effects, but their incidence was not significantly higher in the treated subjects than in the control subjects (Table 6.3). Most reactions were mild and of short duration. Of particular note, no subject reported the development or the worsening of allergic symptoms in temporal association with or following dosing.

6.2.6 Immunogenicity

Most jurisdictions have criteria for licensure of commercial vaccines that are based on clinical surveys comparing serologic and efficacy data. The European Committee on Medicinal Products for Human use (CHMP) has established its own series of criteria which are widely accepted as guidelines for vaccine developers. They are based on three serologic measurements: an HI seroconversion rate of 40 % (i.e., 40 % of subjects have experienced a fourfold rise in titer), a seroprotection rate of 70 % (i.e., 70 % of vaccinated subjects have titers $\geq$1:40), and a geometric mean increase (GMI) in HI titer of $\geq$2.5. For a vaccine to be considered for licensure, it

Table 6.4 Evaluation of the antibody response after first and second dose using three immunological assays

Parameter	H5-VLP vaccine			Placebo
	5 μg	10 μg	20 μg	
3 weeks after first dose (D21)				
HI				
GMT	4.	5.5	7.5	4.5
Number of subject with positive response (%)	8.3	25	50	8.3
Seroprotection (%)	0	0	8.3	0
Seroconversion (%)	0	0	8.3	0
GMI	1.2	1.4	1.7	1.1
SRH				
GMA	9.7	10.0	11.2	6.3
Number of subject with positive response (%)	41.7	33.0	41.7	16.7
Seroprotection (%)	8.3	16.7	25.0	0
Seroconversion (%)	16.7	8.3	25.0	16.7
GMI	1.6	1.3	1.5	1.2
MN				
GMT	6.9	5.0	7.6	5.0
Number of subject with positive response (%)	25	0	33.3	0
Seroconversion (%)	8.3	0	16.7	0
3 weeks after second dose (D42)				
HI				
GMT	11.9	18.2	29.5	4.0
Number of subject with positive response (%)	66.7	100	75	0
Seroprotection (%)	16.7	25.0	50.0	0
Seroconversion (%)	16.7	25.0	58.3	0
GMI	3.0	4.5	6.8	1.0
SRH				
GMA	15.5	16.4	26.2	7.1
Number of subject with positive response (%)	50.0	50.0	75.0	25.0
Seroprotection (%)	41.7	41.7	75.0	8.3
Seroconversion (%)	41.7	50.0	58.3	25.0
GMI	2.5	2.1	3.6	1.3
MN				
GMT	16.8	28.3	48.1	5.7
Number of subject with positive response (%)	83.3	100	91.7	16.7
Seroconversion (%)	41.7	50.0	66.7	0

Note: *HI* hemagglutination inhibition assay, *SRH* single radial hemolysis assay, *MN* microneutralization assay, *GMT* geometric mean titer, *GMI* geometric mean of the increase

generally has to meet or exceed all three criteria. Similar criteria are available for SRH data.

From this study, we saw substantial responses that achieved statistical significance for many comparisons. Depending on the data set used (i.e., HI or SRH), responses to the H5-VLP vaccines met either two or all three of the CHMP criteria (see Table 6.4) at the higher doses. There was a clear dose response between the

5, 10, and 20 μg doses of vaccine. Given the poor immunogenicity of the H5 HA protein, these were among the best responses seen for pandemic H5 vaccines in the industry. Some of these other candidate vaccines had required two doses of 45 μg to achieve a significant response, even with the inclusion of an adjuvant.

6.3 Preparing for the Phase II Trial

Before entering into a Phase II clinical trial, it was decided to revisit sections of the extraction–purification process as there were features that we wanted to change before entering into further clinical development. We wanted first to remove the fetuin affinity step, as we knew that it could pose significant challenges in terms of supply, operations, and sustainability (i.e., the fetuin columns had to be made in-house and were single-use). We also took this opportunity to potentially optimize yields. We knew there were adequate levels of the VLP antigen *in planta*, but our recovery was still unsatisfactory.

We knew VLPs were stacking up between the plasma membrane and the cell wall, and it made no sense that we had to break up the cells to gain access to the VLP. By doing this with harsh mechanical extraction, we were inevitably releasing enormous amounts of host cell material and thus putting undue pressure on downstream processing. In short, by using conventional techniques for extraction, we were not taking advantage of the nature and location of the particles we wanted to purify from the biomass. What we needed was to release the VLPs from the extracellular matrix while avoiding cell breakage.

We thought this would be feasible if depolymerization of the cell wall could be performed by a mild enzyme-assisted approach. Although this was a relatively simple approach from a conceptual perspective, implementing this process at an industrial scale proved to be a challenge. An enzyme-assisted extraction procedure was eventually brought to full-scale operations for 25 kg biomass batches first (H5 pandemic) and then for 350 kg batches for our seasonal program in North Carolina. The use of cell wall-degrading enzymes and mild agitation first liquefied the leaf blades and then released the VLPs at the same time as protoplasts were slowly forming. A series of filtration and continuous-flow centrifugation steps was then developed in order to remove host cells and host cell debris and to prepare the VLP suspension for further purification. As the ensuing VLP preparations were significantly purer at the very beginning of downstream purification than what we had had for the Phase I trial, this improvement alone allowed us to perform final purification with only ion-exchange chromatography and TFF, with no need for the fetuin-based affinity chromatography step.

One striking and gratifying difference was the yield. Analyses revealed that more than 95 % of the released HA was assembled as VLPs by size exclusion chromatography (data not shown). It allowed us to conclude that most of the HA matured as fully assembled trimers and that these trimers migrated to the lipid rafts and budded successfully as VLPs. Thus, it turned out that the process of maturation

and budding was not limiting in any aspect of the production and accumulation of enveloped particles. It was not only a dramatic improvement of the primary recovery itself but also yielded abundant material to enter into the downstream purification processes.

These higher yields also helped in the preparation of clinical lots since single plant biomass batches were now sufficient to produce all of the material needed for a trial, including the test and reserve material. When our engineering runs were over, preparing the vaccine for the preclinical trials including characterization was a matter of only a couple of weeks. These process changes still produced a highly pure preparation with only traces of contaminating proteins, most of them natural membrane proteins. However, since these changes in primary recovery involved a potential source of adventitious agents, special care was taken in the tracking and analysis of contaminants that could have come with the enzyme solutions. It was finally demonstrated that the enzymes were efficiently removed by the early tangential flow filtration step and that they did not contribute to either the final endotoxin or DNA pools.

As changes had been brought to the preparation of the active ingredient, preclinical testing was undertaken to demonstrate comparability. At the time of our meetings with regulatory agencies, four animal studies had been conducted, in mice and ferrets, with safety and immunogenicity as primary endpoints. The active vaccine ingredient produced by this new process was deemed of sufficient quality and potency to be considered for a Phase II study. To further demonstrate efficacy and potency prior to entering in Phase II, a repeat challenge study was performed in ferrets, and a full toxicity study in rats, with essentially identical results to those seen in the earlier studies.

6.3.1 Phase II Clinical Trial of the Plant-Made Influenza VLP Vaccine

The Phase II clinical trial was a randomized, observer-blind, multicenter, placebo-controlled dose-ranging study to evaluate the immunogenicity, safety, and tolerability of two doses of the H5-VLP influenza vaccine (A/Indonesia/5/05) with or without Alhydrogel™ adjuvant in healthy adults 18–60 years of age. The study was divided into two parts. In part A, 135 subjects were randomized into groups of 30 (except the placebo group of 15 subjects) to receive, via IM administration in the deltoid muscle, two doses of 20, 30, or 45 μg H5-VLP vaccine combined with Alhydrogel™, 45 μg of the VLP vaccine without Alhydrogel™, or placebo (PBS) (21 days between doses). Serologic analysis of the day 42 sample suggested that 20 μg H5-VLP combined with Alhydrogel™ was the optimal dose. In part B, an additional 120 subjects were enrolled to evaluate immunogenicity, safety, and tolerability of the 20 μg dose with Alhydrogel (105 subjects) compared to placebo (15 subjects), administered as above. The primary and secondary objectives of both

parts of this study were essentially as described above except that close monitoring of subjects was maintained for 21 days after each dose, and the final assessment occurred 228 days after the first vaccination. Exclusion criteria were essentially the same as described above.

The Phase II clinical trial was approved on October 28, 2010, by BGTD, and the study was conducted in Canada at two sites with shared oversight by an independent review board (IRB Services, Aurora, ON, Canada) and the Research Ethics Board of the McGill University Health Centre (Montreal, QC, Canada). The study was registered at ClinicalTrials.gov under clinical trial registration number NCT01244867. All subjects signed informed consent documents prior to enrollment.

Since plant glycoproteins contain structural motifs (core β1-2 xylose and α1-3 fucose) that are not found on human glycoproteins and as these motifs contribute to IgE and IgG glyco-epitopes on several known plant allergens, one theoretical risk of using plants for the production of biotherapeutics or vaccines was the possible induction of hypersensitivity to plant glyco-epitopes. It was known that many of the glyco-epitopes found on plant allergens shared biochemical and structural homology with glyco-epitopes (cross-reactive carbohydrate determinants or CCDs) found on allergenic glycoproteins from other sources (i.e., from insects, arthropods) and that these CCDs could be involved in the binding of IgE from allergic patients and in the release of histamine by mast cells (Foetisch et al. 2003; Van Ree et al. 2000).

Our analysis of available clinical data showed that roughly 20 % of subjects with pollen and food allergies displayed in vitro CCD reactivity based on β1-2 xylose or α1-3 fucose. As suggestive as these correlations could seem, it had also been shown repeatedly that IgEs directed solely to CCDs typically had little biological activity and no clinical significance even in allergy-prone individuals (van der Veen et al. 1997; Ebo et al. 2004; Altmann 2006). At the initiation of this Phase II study, orally administered, plant-made vaccines had an excellent track record of safety (Tacket 2009).

Our analysis also showed that despite the innocuous nature of most plant glycans, a small number of plant-specific glycan motifs had been associated with the induction of IgE and clinically relevant allergy. Although there were some exceptions (Altmann 2006), the plant glyco-epitopes on these allergens were glycans with core β1-2 xylose and α1-3 fucose and antennae terminated by mannose residues (e.g., MMFX or MUFX). Our detailed analysis of the glycan composition of our H5 vaccine showed that none of these simple MMFX and/or MUXF motifs were present, but rather that most glycosites harbored complex glycans typical of plant glycoproteins. Although our Phase I trial had indicated no links between immunization with the VLP vaccine and either allergic reactions or an immune response to CCDs, these reactions or responses were also monitored during the whole of the Phase II trial.

6.3.2 Immunogenicity and Reactogenicity

As for Phase I, the Phase II subjects were kept under medical observation following immunization and were asked to report symptoms of any perceived health imbalance (solicited or unsolicited). As for Phase I, the plant-derived HA VLP vaccines were well tolerated and induced strong antibody responses against influenza (in preparation). In total, 255 subjects were enrolled in these trials, 218 of whom received at least one dose of a plant-derived VLP vaccine. Five subjects withdrew from the study and were excluded from analyses. Thirteen percent of all subjects reported at least one allergy at recruitment. The most common allergies were "seasonal" (e.g., pollens, ragweed) as well as reactions to penicillin, cats, and dust. No allergic symptoms were reported by any subject in the period immediately following immunization, and no subject declared the onset of new allergies or the worsening of preexisting allergies during the 6-month period following vaccination.

6.3.3 Immune Response to Glycans

Overall, 40 of the 48 subjects with declared allergies had been randomly assigned to VLP treatment groups. Declared allergies to suspected or known plant allergens (e.g., seasonal allergies, hay fever, pollen, ragweed) accounted for almost 50 % of the "allergy-prone" subjects. None of these "allergy-prone" subjects were positive in the bromelain assay at screening, and none mounted any detectable IgE response to the MUXF motif following vaccination. Some of these subjects had increased IgG titers to corn avidin glycans following vaccination, but similar responses were seen in nonallergic subjects (Table 6.5). Two of the subjects with declared allergies, both in VLP-vaccinated groups, mounted IgE responses to corn avidin glycans and VLPs.

Eight subjects enrolled in the Phase II study were positive in the bromelain assay at enrollment, demonstrating that the induction of these potentially worrisome IgE antibodies occurs naturally. Neither of these subjects, all of whom were randomized to VLP vaccination groups, nor the subjects who were bromelain negative at enrollment mounted any IgE response to the MUXF structures following vaccination. Only two of the initially IgE-positive subjects were still positive at 6 months after the last vaccine dose. These observations suggest that, even though plant-specific xylose and fucose motifs are found on the influenza HA contained in our VLP vaccines and are likely present on the trace plant cell protein contaminants as well, these candidate vaccines do not appear to induce IgE responses directed against MUFX motifs in healthy adults.

In contrast to the absence of MUFX responses, transient IgG and IgE responses to the complex glycans found on our VLPs were readily detectable by the corn avidin ELISA following plant-made VLP vaccination (results not shown,

Table 6.5 Antibodies to plant-specific glyco-epitopes

Group	Before immunization (D0)		After two immunizations (D42)			
	Number of subjects with detectable IgGs to plant-specific glyco-epitopes		Number of subject with an increase in IgGs to plant-specific glyco-epitopes		Number of subject with an increase in IgEs to plant-specific glyco-epitopes	
	Total subjects	In subjects who reported known allergy to plant component	Total subjects	In subjects who reported known allergy to plant component	Total subjects	In subjects who reported known allergy to plant component
5 µg H5 VLP	1/12	1/5	2/12	1/5[a]	0/12	0/5
10 µg H5 VLP	3/12	1/3	1/12	0/3	0/12	0/3
20 µg H5 VLP	2/12	0/4	2/12	0/4	0/12	0/4
Placebo	1/12	0/7	1/12	0/7	0/12	0/7
Total	7/48 (14.6 %)	2/19 (10.5 %)	6/48 (12.5 %)	1/19 (5.2 %)	0/48 (0 %)	0/19 (0 %)

[a]Not the same subject who had detectable Abs to plant-specific glycol-epitopes before immunization

manuscript in preparation). However, none of the study subjects with such responses reported allergic-like symptoms, and even those with IgE responses to corn avidin glycans and/or VLPs failed to mount any response to the cross-reactive MUFX motif in the bromelain test. Together, these results suggest that the relatively rare and transient glycan-specific IgE responses induced by our VLP vaccines are unlikely to be targeting CCD motifs. The incidence of glycan-specific IgE responses induced by VLP vaccination did not appear to be higher in the subjects with allergic histories, including those with reported plant allergies.

To our knowledge, this work is the first systematic effort to evaluate the humoral response to the glycans on a plant-made biotherapeutic product. None of the subjects exposed to plant-made VLPs bearing fully glycosylated influenza H5 hemagglutinin proteins from influenza A virus experienced either new-onset allergic symptoms or worsening of preexisting allergies. The absence of response to the known allergy-inducing MMXF/MUFX motifs despite readily detectable IgG and IgE responses to plant glycans following plant-made VLP vaccination was also reassuring. These observations support the continued development of this novel platform for the production of vaccines and other biotherapeutics.

6.4 Further Development

Continued testing of our pandemic H5 vaccine has been aimed at characterizing the strength and breadth of the immune response induced. It has recently been shown that it induces a long-lasting polyfunctional T-cell immune response that is cross-reactive to other influenza strains, which is in contrast with the typical humoral response induced by current split egg-based vaccines. Our result suggests that this broader immune response induced by our VLP might prove more effective than split vaccines in terms of cross-reactivity and offer better protection in the elderly population.

6.5 Concluding Remarks

Since this project was launched, there have been other attempts to develop influenza vaccines using antigens produced in plants. Since HA is a transmembrane protein, there are no simple approaches to the production of soluble HAs; this is true for any recombinant system. HA antigenic domains have been produced as soluble fusion proteins with carriers but not surprisingly, these chimeras proved to have low antigenicity (Musiychuk et al. 2007). The same low antigenicity was observed with the soluble, endoplasmic reticulum-retained HA ectodomain produced by Shoji et al. (2009). As was demonstrated in our own experiments, soluble HAs generally under-perform compared to more structured forms such as VLPs or split vaccines. Other more conserved HA epitopes have also been produced in plants as

chimeras with plant virus capsid proteins, but their potency as vaccines remains to be demonstrated.

Medicago's H5-VLP vaccine program represents a long-awaited breakthrough in the development of plant-based manufacturing technologies for vaccines and has now been accepted by the regulators. The nature of the plant-based transient expression system in itself offers advantages of speed and surge capacity that have no comparator in the traditional biological manufacturing sector. Administered with or without adjuvants (alum, GLA), the H5-VLP vaccine produced by this technology appears to be at least as immunogenic as any other vaccine currently on the market. Our ongoing monitoring of reactivity suggests that even repeated immunizations with high antigen doses and powerful adjuvants can be given safely, even in subjects with a history of allergies to plant materials. To date, we have identified limited and transient IgG and IgE responses to plant glycans in only a minority of subjects following H5-VLP vaccination and no IgE responses to the potentially more pathogenic cross-reactive carbohydrate determinants found on bromelain.

Most importantly perhaps, this vaccine is not just a potential replacement for currently available vaccines. The plant-made H5-VLP vaccine has inherent biological advantages over the vaccines currently used for stockpiling. To date, it is one of the only vaccines that can meet the three CHMP criteria (percentage of fourfold increase in HI titer >40 %, mean geometric increase > 2.5, percentage of HI titer above $1/40 > 70$ %) for licensure at dosages and on a timeline that are compatible with a realistic pandemic preparedness program.

In summary, Medicago's plant-based platform has the potential to dramatically change the prospects of a successful global response to a rapidly moving pandemic, whether H5N1, H7N9, or some other strain. Indeed, the flexibility, rapid response capability, and surge capacity of this new platform would not only be beneficial in influenza outbreaks but could also contribute importantly to addressing a wide range of biological threats.

References

Altmann F (2006) The role of protein glycosylation in allergy. Int Arch Allergy Immunol 142 (2):99–115

Cardineau GA, Mason HS, Vaneck JM, Kirk DD, Wamsley AM (2004) Vector and cells for preparing immunoprotective compositions derived from transgenic plants. International Patent application WO 2004/098533

D'Aoust MA, Lavoie PO, Couture MJ, Trépanier S, Guay JM, Dargis M, Mongrand S, Landry N, Ward BJ, Vézina LP (2008) Influenza virus-like particles produced by transient expression in *Nicotiana benthamiana* induce a protective immune response against a lethal viral challenge in mice. Plant Biotechnol J 6:930–940

D'Aoust MA, Couture MJ, Charland N, Trépanier S, Landry N, Ors F, Vézina LP (2010) The production of hemagglutinin-based virus-like particles in plants: a rapid, efficient and safe responses to pandemic influenza. Plant Biotechnol J 8:607–619

Ebo DG, Hagendorens MM, Bridts CH, De Clerck LS, Stevens WJ (2004) Sensitization to cross-reactive carbohydrate determinants and the ubiquitous protein profilin: mimickers of allergy. Clin Exp Allergy 34(1):137–144

Foetisch K, Westphal S, Lauer I, Retzek M, Altmann F, Kolarich D, Scheurer S (2003) Biological activity of IgE specified for cross-reactive carbohydrate determinants. J Allergy Clin Immunol 111(4):889–896

Gómez-Puertas P, Albo C, Pérez-Pastrana E, Vivo A, Portela A (2000) Influenza virus matrix protein is the major driving force in virus budding. J Virol 74:11538–11547

Landry N, Ward BJ, Trépanier S, Montomoli E, Dargis M, Lapini G, Vézina LP (2010) Preclinical and clinical development of plant-made virus-like particle vaccine against Avian H5N1 influenza. PLoS One 5:e15559, available: http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0015559

Mason HS, Man-Kit LD, Arntzen CJ (1992) Expression of hepatitis B surface antigen in transgenic plants. Proc Natl Acad Sci U S A 89:11745–11749

Musiychuk K, Stephenson N, Bi H, Farrance CE, Orozovic G, Brodelius M, Brodelius P, Horsey A, Ugulava N, Shamloul AM, Mett V, Rabindran S, Streatfield SJ, Yusibov V (2007) A launch vector for the production of vaccine antigens in plants. Influenza Other Respir Viruses 1:19–25

Tacket CO (2009) Plant-based oral vaccines: results of human trials. Curr Top Microbiol Immunol 332:103–117

Van der Veen MJ, van Ree R, Aalberse RC, Akkerdaas J, Koppelman SJ, Jansen HM, van der Zee JS (1997) Poor biologic activity of cross-reactive IgE directed to carbohydrate determinants of glycoproteins. J Allergy Clin Immunol 100(3):327–334

Van Ree R, Cabanes-Macheteau M, Akkerdass J, Milazzo JP, Loutelier-Bourthis C, Rayon C, Villalba M, Koppelman S, Aalberse R, Rodriguez R, Faye L, Lerouge P (2000) β (1,2)-xylose and α (1-3)-fucose residues have a strong contribution in IgE binding to plant glycoallergens. J Biol Chem 275(15):1145–1148

WHO. Availability of a candidate reassortant vaccine virus for the novel influenza A (H1N1) vaccine development. 27 May 2009. Available: http://www.who.int/csr/resources/publications/swineflu/2009_05_271DCDCRG15a.pdf

Wyman O (2007) Influenza strategies for broad global access. Key findings and project methodology. Program for Appropriate Technology in Health (PATH). Available: http://www.oliverwyman.com/ow/pdf_files/VAC_infl_publ_rpt_10-07.pdf

Chapter 7
Plant-Produced Recombinant Transmission Blocking Vaccine Candidates to Combat Malaria

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7.1 Introduction to Malaria Vaccines

Malaria is one of the world's most devastating infectious tropical diseases. It is caused by *Plasmodium* parasites and is transmitted by *Anopheles* mosquitoes. About 219 million clinical cases of malaria were reported worldwide in 2010, predominantly in developing countries in sub-Saharan Africa, causing approximately 660,000 deaths, mostly among children under the age of five years (WHO 2012). In addition to native populations, malaria-naïve travelers and military troops stationed to endemic countries are also at risk. Furthermore, a potential increase in the range of endemic areas due to global warming and climate change may further increase the incidence of malaria (Nabi and Qader 2009). Of the four species of malaria parasites that infect humans, *Plasmodium falciparum* is responsible for the majority of deaths, while *Plasmodium vivax* accounts for over 50 % of all infections outside of Africa as well as 10 % of those in Africa. The symptoms, which often appear about 9–14 days after the infectious mosquito bite, include fever, headache, vomiting, and other flu-like symptoms. If antimalarial drugs are not available or parasites are resistant to them, infection can lead to coma, severe anemia, and death (WHO 2012; Roll Back Malaria 2013). Therefore, reduction in the impact of this disease, and even its eradication, is a commitment of global health and charitable organizations (WHO 2012).

Control of malaria is not imminent. Currently, measures to control transmission are limited to the use of insecticide-treated bed nets in endemic regions and indoor residual spraying with insecticides to restrict the number of infectious bites, thereby reducing morbidity and mortality (Lengeler 2004). In addition, travelers to endemic countries are advised to take antimalarial prophylactic drugs (CDC 2012). Chemotherapy is the only available treatment for infected individuals (WHO 2012), but chemical control measures often fail (Enserink 2010; Good et al. 1998), and

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increasing frequency of drug resistance of the parasite makes effective chemotherapy challenging (WHO 2010, 2012). Therefore, there is an urgent need for alternative strategies to combat malaria, such as effective vaccines to prevent infection and spread of the disease.

No licensed vaccine for prophylaxis against malaria is currently available. However, significant efforts are under way to identify promising antigens and epitopes to develop recombinant vaccines. Targets of potential malaria vaccines include pre-erythrocytic stages (infective sporozoites and replicating liver-stage parasites), erythrocytic stages (replicating merozoites and gametocytes), and mosquito, or sexual, stages (gametes, zygotes, and ookinetes). Vaccines targeting pre-erythrocytic, erythrocytic, and sexual stages of the *Plasmodium* parasite's life cycle are directed toward prevention of infection, clinical illness, and disease transmission, respectively (Hill 2011; Thera and Plowe 2012). The global malaria vaccine pipeline, presented in the World Health Organization (WHO) rainbow chart (WHO 2013), covers these approaches, which are reviewed by Schwartz et al. (2012) and summarized in Table 7.1. Malaria vaccine candidates that have entered clinical trials include whole organism (sporozoite)-based vaccines targeting the pre-erythrocytic stage; recombinant non-replicating viral vector-based vaccines targeting the erythrocytic stage; DNA vaccines targeting the pre-erythrocytic stage; subunit vaccines targeting the pre-erythrocytic, erythrocytic, and sexual stages; and combination vaccines (Table 7.1).

A prominent vaccination approach utilizing the whole *Plasmodium* organism is based on multiple studies demonstrating that high-level, sustained, protective immunity against the pre-erythrocytic stages of *P. falciparum* can be induced by immunization through the bites of >1,000 irradiated mosquitoes carrying *P. falciparum* sporozoites (PfSPZ) (Clyde 1990; Rieckmann 1990). Consequently, an injectable pre-erythrocytic vaccine candidate, Sanaria® PfSPZ, composed of live non-replicating (radiation-attenuated), aseptic, purified, cryopreserved PfSPZ, has been developed and produced under current good manufacturing practice (cGMP) guidelines by Sanaria Inc. (Rockville, MD) (Hoffman et al. 2010). Initially, the vaccine was administered subcutaneously or intradermally and was shown to elicit low immune responses and minimal protection (Epstein et al. 2011). Recently, however, results of another Phase 1 clinical trial have been published, demonstrating that five doses of Sanaria® PfSPZ administered intravenously can provide complete protection against malaria in subjects exposed to *P. falciparum* parasites. Vaccinated subjects developed both PfCSP- and PfSPZ-specific antibody titers and T cell responses, which were dose dependent (Seder et al. 2013). Despite the demonstrated efficacy of Sanaria® PfSPZ, this approach may be problematic at large scale because of vaccine production costs and the impracticality of intravenous injections as a route of inexpensive vaccine administration.

By contrast, viral vectors used for the development of malaria vaccines include non-replicating simian adenovirus (AdCh63) and modified vaccinia virus Ankara (MVA). In a Phase 1a clinical trial, priming with AdCh63 expressing fragments of merozoite surface protein 1 (MSP-1), the erythrocytic-stage antigen of *P. falciparum*, was followed by boosting with MVA expressing the same antigen. This heterologous

Table 7.1 Summary of malaria vaccination strategies and candidate vaccines

Vaccination strategy	Parasite stage	Candidate vaccine	Type of vaccine	Antigen	Expression system	Adjuvant	Clinical development
Block sporozoite invasion of and division in liver cells	Pre-erythrocytic	RTS,S	Subunit vaccine (HBsAg VLP)	CSP	*S. cerevisiae*	AS01	Phase 3
		Ad35.CS	Non-replicating virus	CSP	Vaccinee	None	Phase 1b
		Ad35.CS prime RTS,S boost	Non-replicating virus Subunit vaccine (HBsAg VLP)	CSP CSP	Vaccinee *S. cerevisiae*	AS01	Phase 1/2a
		AdCh63 ME-TRAP prime MVA ME-TRAP boost	Non-replicating viruses	ME and TRAP	Vaccinee	None	Phase 2
		Polyepitope DNA EP1300	DNA	CSP, SSP2/TRAP, LSA-1, and Exp-1	Vaccinee	None	Phase 1a
		Sanaria® PfSPZ	Whole parasite	Multi-antigen	Sporozoite	None	Phase 1/2a
		Genetically attenuated sporozoites	Whole parasite	Multi-antigen	Sporozoite	None	Phase 1
Block merozoite invasion of and division in erythrocytes and block merozoite differentiation into gametocytes	Erythrocytic	AdCh63 MSP-1 prime MVA MSP-1 boost	Non-replicating viruses	MSP-1 MSP-1	Vaccinee	None	Phase 2a
		FMP010	Subunit vaccine (protein)	MSP-1	*E. coli*	AS01B	Phase 1b
		MSP-3	Subunit vaccine (protein)	MSP-3	Synthesized in vitro	Aluminum hydroxide	Phase 2
		FMP2.1	Subunit vaccine (protein)	AMA-1	*E. coli*	AS02A	Phase 1b, 2
		FMP2.1	Subunit vaccine (protein)	AMA-1	*E. coli*	AS01B	Phase 1/2a

(continued)

Table 7.1 (continued)

Vaccination strategy	Parasite stage	Candidate vaccine	Type of vaccine	Antigen	Expression system	Adjuvant	Clinical development
		AMA-C1	Subunit vaccine (protein)	AMA-1	*P. pastoris*	Alhydrogel and CpG 7090	Phase 1b, 2
		AdCh63 AMA-1 prime MVA AMA-1 boost	Non-replicating viruses	AMA-1	Vaccinee	None	Phase 1a, 2a
		EBA175 RII	Subunit vaccine (protein)	EBA175	*P. pastoris*	Aluminum phosphate	Phase 1
		SE36	Subunit vaccine (protein)	SERA5	*E. coli*	Aluminum hydroxide	Phase 1b
		BSAM-2	Subunit vaccine (proteins)	MSP-1 and AMA-1	*E. coli*, *P. pastoris*	Alhydrogel and CpG 7090	Phase 1b
		JAIVAC	Subunit vaccine (proteins)	MSP-1 (19) and EBA175	*E. coli*	Montanide ISA 720	Phase 1a
		GMZ2	Subunit vaccine (protein)	MSP-1-GLURP fusion	*L. lactis*	Aluminum hydroxide	Phase 1b Phase 2b
Combination (multistage)	Pre-erythrocytic and erythrocytic	PEV301 PEV302	Subunit vaccines (peptides on virosomes)	CSP AMA-1	Synthesized in vitro	None	Phase 1b, 2a
Block parasite sexual development in mosquito and parasite transmission	Sexual	Pfs25 Pvs25	Subunit vaccines (proteins)	Pfs25 Pvs25	*P. pastoris* *S. cerevisiae*	Montanide ISA 51	Phase 1 (terminated)
		Pfs25 conjugated to EPA	Subunit vaccine (protein)	Pfs25	*S. cerevisiae*	Alhydrogel	Phase 1a

Ad adenovirus, *AMA-1* apical membrane antigen 1, *CSP* circumsporozoite protein, *EPA* ExoProtein A of *Pseudomonas aeruginosa*, *Exp-1* exported protein 1, *GLURP* glutamate-rich protein, *HBsAg VLP* hepatitis B surface antigen virus-like particle, *LSA-1* liver-stage antigen 1, *ME* multiple epitopes, *MSP* merozoite surface protein, *MVA* modified vaccinia virus Ankara, *TRAP* thrombospondin-related adhesion protein

prime-boost vaccination induced very high mixed CD4/CD8 T cell responses and substantial MSP-1-specific IgG responses that, however, did not reach titers sufficient to neutralize *P. falciparum* in vitro (Sheehy et al. 2011). Similar results were obtained in another Phase 1a trial where AdCh63 and MVA encoding two alleles (3D7 and FVO) of apical membrane antigen 1 (AMA-1), the erythrocytic-stage antigen of *P. falciparum*, were administered in a prime-boost fashion. In this trial, in addition to strong T cell responses against both alleles with a mixed CD4/CD8 phenotype, substantial AMA-1-specific serum IgG responses demonstrated an inhibitory activity against *P. falciparum* in vitro (Sheehy et al. 2012).

The development of subunit vaccines is the most intensively pursued strategy for producing a malaria vaccine and is based on peptides, soluble proteins, and virus-like particles (VLPs) produced in heterologous expression systems or synthesized in vitro. For example, FMP2.1, targeting the erythrocytic stage of the parasite, represents the ectodomain of AMA-1 of *P. falciparum* and has been produced in and purified from *Escherichia coli* at the Walter Reed Army Institute of Research Pilot Bioproduction facility (Forest Glen, Maryland). When administered with the liposomal formulation adjuvant AS02(A), this antigen was shown to be immunogenic in African children and to reduce the risk of clinical malaria caused by parasites that have a corresponding AMA-1 by more than 60 % (Thera et al. 2010, 2011).

As an example of a synthetic subunit vaccine, PEV3, produced by Pevion Biotech (Ittigen, Switzerland), is a bivalent VLP-based vaccine displaying two synthetic peptides from circumsporozoite protein (CSP) and AMA-1 on the surface of influenza virosomes. PEV3 targets both pre-erythrocytic and erythrocytic stages and has been shown to induce long-lasting sporozoite-inhibitory antibody titers in Phase 1a/b clinical trials (Genton et al. 2007; Okitsu et al. 2007; Cech et al. 2011).

The most advanced malaria vaccine under development is RTS,S, developed by GlaxoSmithKline Biologicals (Rixensart, Belgium), with additional support from the Program for Appropriate Technology in Health and the Bill and Melinda Gates Foundation. RTS,S targets the pre-erythrocyte stage of parasite development and comprises *Saccharomyces cerevisiae*-produced recombinant VLPs in which hepatitis B surface antigen (HBsAg) is fused to CSP (Stoute et al. 1997). Recently, a Phase 3 clinical trial of RTS,S adjuvanted with AS01 was conducted in 15,000 children in two age categories, 6–12 weeks and 5–17 months, at 11 centers in seven malaria-endemic sub-Saharan African countries. Interim results of the first year showed a vaccine efficacy for RTS,S/AS01 of 55.8 % (97.5 % confidence interval [CI], 50.6–60.4) in the older per-protocol population and a vaccine efficacy of 34.8 % (95 % CI, 16.2–49.2) in the combined age categories per-protocol population. The vaccine was shown to be safe, with a frequency of serious adverse events in both age groups similar to subjects that received an unrelated licensed vaccine. The most frequently reported symptoms were pain and fever (RTS,S Partnership 2011). The second year interim results for 6,537 infants at 6–12 weeks of age, immunized in conjunction with the Expanded Program on Immunization, demonstrated that the per-protocol efficacy for the prevention of first or only episodes of malaria during the 12 months after the last vaccination was 31.3 % and the efficacy

against severe malaria was 36.6 % (RTS,S Partnership 2012). In addition, an extended follow-up of a Phase 2b RTS,S/AS01E trial in Kenya demonstrated that the efficacy of RTS,S/AS01E against first and all episodes of *P. falciparum* clinical malaria waned during 4 years of follow-up to levels of 29.9 % and 16.8 %, respectively, among children vaccinated at 5–17 months of age (Olotu et al. 2013). A Phase 3 trial of RTS,S/AS01E will continue through 2014 and will report the effect of a booster dose of RTS,S/AS01 in some participants at 18 months after the initial immunization, as well as the overall duration and magnitude of protection against clinical and severe malaria. Based on available data, GlaxoSmithKline intends to submit a regulatory application to the European Medicines Agency (EMA) in 2014, and the World Health Organization (WHO) indicates that a policy recommendation for the RTS,S malaria vaccine candidate is possible as early as 2015 if it is granted a positive scientific opinion by EMA (PATH MVI 2013). However, the above reports of limited efficacy with this and other pre-erythrocytic- and blood-stage vaccine candidates have prompted the exploration of alternative strategies.

7.2 Transmission Blocking Vaccines for Targeting Disease Reduction and Eradication

Malaria transmission blocking vaccines (TBVs) are proposed as an effective means to reduce malaria transmission (WHO 2000) and are considered an important element in eventually eradicating the disease (WHO 2000). This strategy relies on a vaccinated individual raising antibodies against proteins displayed during the sexual stages of parasite development. During a blood meal, these antibodies are ingested by the mosquito vector along with any *Plasmodium* gametes that may be in the blood stream. The antibodies then prevent the development of oocysts in the mosquito midgut by binding to the surface proteins of gametocytes, gametes, zygotes, and/or ookinetes, thus inhibiting sexual reproduction of the parasite (Aly et al. 2009). This mechanism blocks sexual maturation into infective sporozoites and prevents transmission of the parasite to the next human host during the mosquito's subsequent blood meals.

Several TBV candidates are under development, the most advanced of which have entered Phase 1 clinical trials. The leading TBV candidates elicit antibodies against the *P. falciparum* gametocyte and gamete antigens Pfs48/45 and Pfs230 or against the *P. falciparum* zygote and ookinete antigens Pfs25 and Pfs28 (Vermeulen et al. 1985; Quakyi et al. 1987; Williamson et al. 1995; Duffy and Kaslow 1997; Pradel 2007). The *P. vivax* Pvs25, Pvs28, and Pvs230 antigens also show promise as vaccine candidates (Hisaeda et al. 2000; Tachibana et al. 2012). Figure 7.1 shows the stages of *Plasmodium* parasite development in the mosquito midgut and at what point TBV targets are expressed. P48/45 and P230 are expressed on gametocytes developing in the human bloodstream, in contrast to

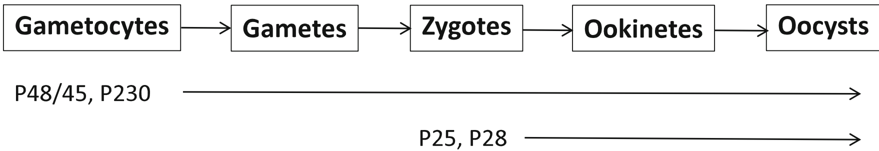

Fig. 7.1 Stages of malaria parasite development in the mosquito midgut and timing of TBV candidate target expression

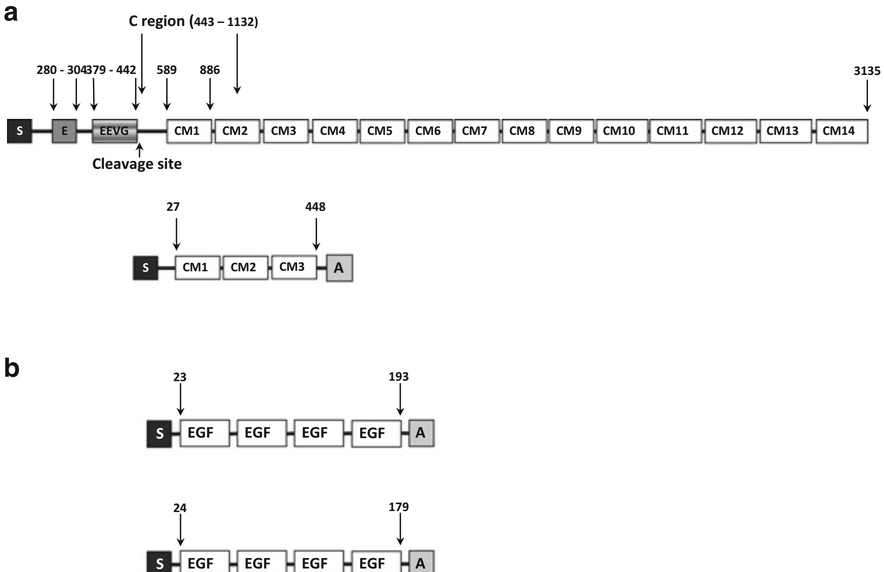

Fig. 7.2 Schematic representation of the repetitive domain structure of *P. falciparum* sexual-stage surface antigens explored as TBV targets. (**a**) Molecules belonging to the 6-cysteine domain family. The cleaved portion of Pfs230 contains the 25 glutamic acid repeat region E (amino acids 280–304) and the 16 tandem EEVG repeat region (amino acids 379–442). A functional domain C spans amino acids 443–1132. (**b**) Molecules belonging to the epidermal growth factor (EGF)-like domain family. *A* anchor domain, *CM* 6-cysteine motif domain, *S* signal peptide

P25 and P28, which are never expressed in the human host. Therefore, P48/45 and P230 are able to naturally self-boost immunity after a repeated infection of the human host with the malaria parasite (Graves et al. 1988; Bousema et al. 2006; Ouédraogo et al. 2011) or presumably after infection of the human host with the parasite following vaccination with a TBV including P48/45 or P230.

The production of P230 and P48/45 candidate antigens in recombinant systems has been very challenging. The presence of cysteine-rich motifs with extensive disulfide bridges (Fig. 7.2a) (Carter et al. 1995; Gerloff et al. 2005) and the lack of N-linked glycosylation of the targets in *Plasmodia* (Samuelson et al. 2005) has made expression in typical prokaryotic and eukaryotic hosts, respectively, problematic. For example, Pfs48/45 contains one and a half of the cysteine-rich double

domains typical in gamete surface proteins as well as seven putative N-glycosylation sites (Kocken et al. 1993; Carter et al. 1995), making production of correctly folded recombinant Pfs48/45 displaying transmission blocking epitopes very difficult. Pfs48/45 plays a key role in fertilization (van Dijk et al. 2001), and antibodies raised against this antigen prevent the development of the zygote and transmission through mosquitoes (Outchkourov et al. 2007, 2008). The major epitopes of Pfs48/45 inducing transmission blocking antibodies (epitopes I and III) are localized within a region containing ten cysteine residues comprising the middle (4-cysteine) and C-terminal (6-cysteine) domains (Outchkourov et al. 2007). Pfs48/45 has been produced in the *Pichia pastoris* expression system. However, the expressed protein did not have transmission blocking activity and did not induce transmission blocking antibodies in mice (Milek et al. 2000). Subsequently, a properly folded, immunogenic Pfs48/45 C-terminal subdomain containing transmission blocking epitopes (Outchkourov et al. 2007) fused to maltose-binding protein was produced in *E. coli* by co-expression with four periplasmic folding chaperones (Outchkourov et al. 2008). The fusion antigen induced uniform and high antibody titers in mice and elicited functional transmission blocking antibodies in 90 % of immunized animals (Outchkourov et al. 2008). Clinical evaluation of this TBV candidate is under way (CORDIS 2012). Another approach focused on harmonizing the codon usage frequency of the target gene with that of the expression host to produce correctly folded, full-length, recombinant Pfs48/45 in *E. coli*. In the presence of an adjuvant such as alum, Montanide ISA-51, or complete Freund's, the recombinant Pfs48/45 antigen induced strong antibody responses and potent transmission blocking activity in mice and nonhuman primates (Chowdhury et al. 2009).

The P230 antigen is a target for TBV development because of a positive correlation between the activity of sera of malaria patients to bind P230 and serum transmission blocking activity (Graves et al. 1988; Healer et al. 1999). Expression of recombinant full-length Pfs230 has not been accomplished due to the large size and high degree of complexity of the target (Carter et al. 1995; Williamson et al. 1995; Gerloff et al. 2005). However, a functional domain C spanning amino acids 443–1132 of Pfs230, starting within the EEVG tetramer region of the protein and continuing to the middle of the second cysteine motif domain (Fig. 7.2a), has been expressed as a fusion to the maltose-binding protein in *E. coli*. This antigen elicited transmission blocking antibodies that, in the presence of complement, partially reduced the infectivity of *P. falciparum* to mosquitoes (Williamson et al. 1995).

Pfs25 is a member of the P25 family of proteins characterized by the presence of epidermal growth factor (EGF)-like repeat motifs and numerous cysteine residues (Fig. 7.2b) resulting in a complex tertiary structure (Kaslow et al. 1988). Consequently, these proteins are difficult to produce with an accurate conformation in recombinant systems. Despite this challenge, recombinant versions of Pfs25 have been expressed in yeasts, and their potential as TBV candidates has been demonstrated. Recombinant Pfs25 was first produced in *S. cerevisiae* as a truncated protein, with mutations in two N-linked glycosylation sites and with a hexa-

histidine (6xHis) tag to facilitate purification (Barr et al. 1991). Although the yeast-produced protein was not recognized by conformation-dependent neutralizing monoclonal antibodies specific to Pfs25, it elicited a strong antibody response with transmission blocking activity in mice and nonhuman primates when administered with Freund's adjuvant or the oil-in-water adjuvant MF59 (Barr et al. 1991). Additional studies confirmed the requirement for adjuvants to elicit strong and long-lasting immunity (Kaslow et al. 1994). Expression and purification of Pfs25 was subsequently optimized in *S. cerevisiae*, and expression was boosted by switching to *P. pastoris* as the host (Zou et al. 2003).

P28 is structurally similar to P25 (Fig. 7.2b), the genes encoding these proteins are genetically linked on chromosome 10 of *P. falciparum* (Duffy and Kaslow 1997), and the proteins have multiple and partially redundant functions (Tomas et al. 2001). Recombinant Pfs28, produced in *S. cerevisiae*, elicited antibodies in mice that blocked transmission of *P. falciparum* and acted synergistically with anti-Pfs25 antibodies when injected into mice in the presence of alum adjuvant (Duffy and Kaslow 1997). Similarly, Pvs25 and Pvs28 of *P. vivax* produced in *S. cerevisiae*, administered with aluminum hydroxide adjuvant, elicited antibodies in mice that completely blocked both the development of *P. vivax* and its ability to infect mosquitoes (Hisaeda et al. 2000).

Pfs25- and Pvs25-based TBV candidates have been evaluated in clinical trials. In a Phase 1 study of a Pvs25-based candidate produced in *S. cerevisiae* and adsorbed onto Alhydrogel®, anti-Pvs25 antibody levels peaked after the third vaccine dose and had significant transmission blocking activity as assessed using the standard membrane feeding assay (SMFA) (Malkin et al. 2005). In a separate Phase 1 study, recombinant Pfs25 and Pvs25 produced in *P. pastoris* and *S. cerevisiae*, respectively, were administered with the water-in-oil adjuvant Montanide ISA 51. Three groups of volunteers received 5 μg of Pfs25/ISA 51, 5 μg of Pvs25/ISA 51, or 20 μg of Pvs25/ISA 51, and a fourth group received adjuvant control (phosphate buffered saline/ISA 51). The trial was terminated prematurely due to the incidence of systemic adverse reactions (erythema nodosum) in some subjects in the group that received 20 μg of Pvs25/ISA 51, considered to be caused by the specific Pvs25 antigen/adjuvant combination and not by the antigen alone (Wu et al. 2008). However, analysis of sera from volunteers who completed two scheduled doses of Pfs25/ISA 51 showed significant levels of anti-Pfs25 antibody titers 30–60 days after the second vaccination and detectable antibody levels one year after the second vaccination. As shown previously in preclinical work (Miura et al. 2007), anti-Pfs25 antibody titers correlated with transmission blocking activity of antisera in SMFA (Wu et al. 2008).

Subsequently, Pfs25 and Pfs28 were chemically conjugated to a mutant, nontoxic ExoProtein A (EPA) of *Pseudomonas aeruginosa* resulting in a significant increase in immunogenicity and transmission blocking activity in mice compared to the unconjugated antigens, especially when adsorbed onto aluminum hydroxide (Kubler-Kielb et al. 2007; Qian et al. 2007, 2008, 2009). The conjugate appears as a nanoparticle of approximately 20 nm (Shimp et al. 2013). An open-label, dose-escalating, Phase 1 clinical trial with the Pfs25-EPA/Alhydrogel® vaccine

candidate (NCT01434381), enrolling 30 healthy malaria-naïve subjects and sponsored by the National Institute of Allergy and Infectious Diseases, began in August 2011 and is estimated to be completed in January 2014 (ClinicalTrials.gov 2013a). Study subjects received up to three doses of 8, 16 (at 0 and 2 months), or 47 μg (at 0, 2, and 4 months) of Pfs25. Interim results demonstrate that the majority of solicited adverse events were mild in severity, the most common solicited adverse event was pain at the injection site, and the frequency of adverse events decreased with each successive dose of vaccine. No vaccine-related serious adverse events have been reported. The vaccine was more immunogenic with each successive dose. In the 47-μg vaccine dose group, after the second and third vaccinations, respectively, geometric mean antibody levels were 92 enzyme-linked immunosorbent assay (ELISA) units (95 % CI, 55–155) and 228 ELISA units (95 % CI, 151–344), and antibody responses were detected in 16 of 17 subjects and 15 of 15 subjects, respectively. Furthermore, Pfs25-EPA/Alhydrogel®-induced antibodies exhibited transmission blocking activity, as demonstrated by SMFA (Talaat et al. 2012).

Despite these successes, there is a pressing need for more economical production of P25, P28, P48/45, and P230 vaccine candidates in order to develop vaccines that will be widely available for developing countries that bear the vast majority of the disease burden. This has stimulated activity in expressing these targets in plant and algal production systems.

7.3 Plant- and Algae-Based Production of TBV Candidates

7.3.1 Potential Advantages of Plant and Algal Systems for the Production of TBV Antigens

During the last two decades, the potential of plants as platforms for the production of recombinant vaccines and therapeutic proteins has been demonstrated in numerous studies (Rybicki 2010; Yusibov et al. 2011). In contrast to prokaryotic and other eukaryotic expression systems, plants combine cost-effectiveness, high scalability, and product safety due to the lack of harbored microbial toxins or mammalian pathogens (Mett et al. 2008a). In addition, plants are able to perform eukaryotic posttranslational modifications of target proteins, particularly N-linked glycosylation, which is shared with mammalian cells, although some differences in glycan decoration exist and may require humanization of specific posttranslational modifications for some applications (Gomord et al. 2010). Some vaccine candidates produced in both transgenic and transiently transformed plant systems have reached clinical or advanced preclinical stages of development (reviewed by Yusibov et al. 2011). One product, taliglucerase alfa, produced by Protalix BioTherapeutics Inc. (Carmiel, Israel) in transgenic carrot cell culture as an enzyme replacement therapy for Gaucher disease, has been approved for marketing by the US Food and Drug Administration (FDA) (Shaaltiel et al. 2007; ASHSP 2012).

Plant-based subunit vaccine antigens are expressed in either transgenic or transplastomic whole plants or cell suspensions or in transient expression systems. The latter utilize plants that are not genetically modified but are infiltrated with plant viral vectors, *Agrobacterium*-based binary vectors, or hybrid vectors that combine features of viral and binary vectors (reviewed by Yusibov et al. 2013). Fraunhofer USA Center for Molecular Biotechnology has developed one such hybrid expression system in which plants are infiltrated with recombinant *Agrobacteria* harboring a "launch vector" combining genetic elements of a plant viral vector derived from *Tobacco mosaic virus* (TMV), where the viral coat protein is substituted with target gene sequence, and a binary vector to allow for propagation in Agrobacteria and delivery to plant cells (Musiychuk et al. 2007). This technology allows for rapid, transient, high-level production of recombinant proteins in hydroponically grown, genetically unmodified *Nicotiana benthamiana* plants and has successfully been used to produce immunogenic subunit vaccine candidates against anthrax, plague, and influenza, among other disease targets (Chichester et al. 2007, 2009; Mett et al. 2008b; Shoji et al. 2008, 2009a, b, 2011). Two of these products have reached clinical development (Chichester et al. 2012; Cummings et al. 2014) and attest to the efficacy of the system.

Unicellular eukaryotic green algae represent an alternative platform to terrestrial plants for the production of recombinant proteins. Rapid nuclear and chloroplast transformation of microalgae into stable lines, safety of algal products, advances in engineering of photosynthesis and cell metabolism, and the ability to grow in contained photobioreactors with controlled culture conditions make the microalgae platform a highly promising biotechnological approach (Rosenberg et al. 2008; Ugwu et al. 2008). Furthermore, the ability of chloroplasts for complex protein folding and disulfide bond formation in the absence of posttranslational N-linked protein glycosylation (Nugent and Joyce 2005) is advantageous for the production of recombinant proteins that do not display N-linked glycans in their native host, such as *Plasmodium* antigens (Samuelson et al. 2005). The most commonly used species of algae, *Chlamydomonas reinhardtii*, has been used to produce monoclonal antibodies (Tran et al. 2009), therapeutic proteins (Rasala and Mayfield 2011), and subunit vaccines (Dauvillée et al. 2010) in chloroplasts.

7.3.2 Production of Recombinant Transmission Blocking Vaccine Candidates in Algae

Recently, successful studies have been published on the production of TBV candidates in microalgae. Gregory et al. (2012) demonstrated that non-glycosylated Pfs25 and Pfs28 antigens, structurally similar to the native proteins and produced in *C. reinhardtii*, could be accumulated in transformed chloroplasts at levels of 0.5 % and 0.2 % of total soluble protein, respectively, as determined by ELISA analysis. Target proteins, which were codon optimized for expression in chloroplasts, each

containing four EGF-like domains and a C-terminal purification FLAG tag but no native signal sequence or the glycosylphosphatidylinositol (GPI) anchor sequence, were purified by affinity chromatography using anti-FLAG M2 affinity resin, and the products were confirmed by mass spectrometry. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analyses showed single predominant bands of reduced Pfs25 and Pfs28 migrating near their predicted sizes of 21.4 kDa and 20.2 kDa, respectively, with few obvious impurities for either target (Gregory et al. 2012). The apparent larger sizes of Pfs25 and Pfs28 observed in both non-reduced immunoblots and native Coomassie-stained SDS-PAGE gels were suggested to result from assembly of monomers into higher molecular weight aggregates due to interactions between beta-strand secondary structures present in both proteins (Richardson and Richardson 2002). Target proteins displayed correct conformations, as shown by immunoblotting with epitope-specific monoclonal antibodies and by circular dichroism spectroscopy. Mass spectrometry analysis of free cysteine residues in algal Pfs25 versus yeast-produced Pfs25 showed formation of disulfide bonds 1, 4, and 6 in algal Pfs25, while bonds 2, 7, 8, 10, and 11 were not completely formed, and peptides for the remaining disulfide bonds were not detected (Gregory et al. 2012). Thus, disulfide bonds in algal Pfs25 formed to a lesser extent than in yeast-derived Pfs25. Serum antigen-specific IgG antibody titers and transmission blocking activity were evaluated in mice immunized intraperitoneally with 25 μg of purified algal Pfs25 or Pfs28 with complete Freund's adjuvant followed by four boosters at 2-week intervals with 15 μg of antigen with incomplete Freund's adjuvant. The results demonstrated that algae-produced Pfs25 and Pfs28 each elicited high titers of antibodies that were able to bind to the surface of cultured sexual-stage malaria parasites. Furthermore, anti-Pfs25 antisera completely blocked parasite transmission ($p < 0.01$). By contrast, anti-Pfs28 antisera exhibited partial transmission blocking activity that was not significant ($p > 0.1$) (Gregory et al. 2012). The duration of transmission blocking activity in sera of immunized animals was not assessed in this study.

In a subsequent study, Pfs25 was produced in algal chloroplasts as a fusion to cholera toxin B subunit (CTB), which is sometimes used as a mucosal adjuvant, and the potential of the fusion protein as an oral vaccine against malaria was examined in mice. Oral vaccination with a relatively impure protein product would potentially greatly reduce production, storage, and administration costs of a vaccine. However, while oral vaccination with the Pfs25-CTB fusion elicited anti-CTB and anti-Pfs25 secretory IgA antibodies, no serum IgG antibody against Pfs25 associated with transmission blocking activity was induced, indicating that while CTB is known to be effective for mucosal vaccines against pathogens that invade mucosal surfaces, it may not be effective in helping malaria antigen candidates to elicit antibodies (Gregory et al. 2013).

The algal system has also successfully been used to produce Pfs48/45. The C-terminal region of Pfs48/45, containing epitopes responsible for induction of transmission blocking activity, was engineered with sequence encoding the GPI domain and a FLAG purification tag and was expressed in chloroplasts of *C. reinhardtii*. The truncated design resulted in increased stability of algal Pfs48/45 and decreased disulfide bridge formation. However, recombinant protein was

predominantly insoluble in aqueous solution, likely due to incorporation into the chloroplast membrane (Jones et al. 2013b). Importantly, epitopes associated with transmission blocking activity were correctly folded in *C. reinhardtii*-produced Pfs48/45 as confirmed by recognition by an established anti-Pfs48/45 conformation-specific transmission blocking antibody (Carter et al. 1990) in both immunoblot and ELISA analyses (Jones et al. 2013b). Immunogenicity and protective efficacy of the algal Pfs48/45 TBV candidate has not yet been reported.

The successful expression of Pfs25, Pfs28, and Pfs48/45 TBV candidates, as well as the erythrocytic-stage target antigens AMA-1 and MSP-1 (Dauvillée et al. 2010), in chloroplasts of *C. reinhardtii* demonstrates the potential of algae as a platform to produce effective, cost-efficient malaria vaccines (Jones and Mayfield 2013).

7.3.3 Production of Recombinant Transmission Blocking Vaccine Candidates in a Transient Plant Expression System

The production of malaria subunit vaccine candidates has also been demonstrated in plants. Specifically, the launch vector-based transient expression technology (Musiychuk et al. 2007) has been applied to produce recombinant Pfs48/45, Pfs230, Pfs25, and Pfs28 in *N. benthamiana*, and these molecules are being evaluated for further development as TBV candidates.

7.3.3.1 Pfs48/45

Pfs48/45, excluding its native signal sequence and C-terminal GPI anchor, has been expressed in *N. benthamiana* with a 6xHis purification tag followed by the endoplasmic reticulum retention signal (KDEL) at the C-terminus, using the launch vector expression system. However, expression levels and solubility were relatively low, and the purified molecule had relatively poor transmission blocking activity (Mamedov et al. 2012). In the native host, Pfs48/45 undergoes complex posttranslational modifications, but there is no N-linked glycosylation (Milek et al. 1998). However, Pfs48/45 contains potential N-linked glycosylation sites that can be aberrantly glycosylated during protein expression in mammalian, fungal, or plant systems, potentially resulting in masking of epitopes, improper folding, and impaired biological activity. To produce a non-glycosylated form of Pfs48/45 in plants, target antigen was transiently co-expressed in *N. benthamiana* with bacterial PNGase F (peptide:N-glycosidase F) of a Gram-negative bacterium, *Flavobacterium meningosepticum* (Mamedov et al. 2012). PNGase F cleaves a bond between the innermost GlcNAc and asparagine residues of high-mannose, hybrid, and complex oligosaccharides in N-linked glycoproteins, except when the

α(1–3) core is fucosylated (Plummer et al. 1984; Tarentino et al. 1990). The in vivo deglycosylated Pfs48/45 protein retained its native conformation, as shown by much stronger recognition of deglycosylated compared to glycosylated Pfs48F1 by monoclonal antibodies I, III, and V raised against various epitopes (I, III, and V) of native Pfs48/45 of *P. falciparum* (Mamedov et al. 2012). This *in planta* deglycosylated version of Pfs48/45 has not yet been assessed for transmission blocking activity.

7.3.3.2 Pfs230

Due to its large size and complex repetitive structure, the focus in plants has been on expressing regions of Pfs230 that have been identified as potential vaccine candidates. The full-length C region of Pfs230 and its truncated forms, Pfs230C0 (residues 443–588), Pfs230C1 (443–715), and Pfs230C2 (443–915) (Fig. 7.2a), have been produced in a wheat germ cell-free expression system (Tsuboi et al. 2008), and rabbit antibodies raised against these recombinant proteins showed significant transmission blocking activity (Tachibana et al. 2011). Since large-scale manufacturing using the wheat germ cell-free expression system is not practical and to date there is no cGMP-compliant wheat germ cell-free facility, a portion of Pfs230 domain C (residues 444–730), 230CMB, has been engineered and produced in *N. benthamiana* using the launch vector. The target protein, containing the endoplasmic reticulum retention signal KDEL and a 6xHis affinity purification tag at the C-terminus, was expressed in *Agrobacterium*-infiltrated *N. benthamiana* plants at approximately 800 mg/kg of fresh leaf tissue and showed approximately 100 % solubility. A downstream process was developed that included immobilized metal ion affinity chromatography (IMAC) and anion exchange chromatography and resulted in recovery of 95 % pure antigen. The purified antigen preparation comprised a single target species of approximately 48 kDa as assessed by SDS-PAGE under reducing conditions, although the nominal molecular mass is 34 kDa. This difference in the molecular mass was shown to be, at least in part, due to glycosylation. Furthermore, size exclusion chromatography (SEC) coupled with multi-angle laser light scatter (MALLS) analysis demonstrated that 230CMB exists in mostly monomeric form, with a very small amount of a high molecular weight species in the protein sample. Plant-produced 230CMB displayed transmission blocking epitopes as demonstrated by its recognition by anti-Pfs230 transmission blocking antibodies in an ELISA. Evaluation of immunogenicity and transmission blocking activity of the plant-produced Pfs230 vaccine candidate showed that a 100-μg dose of 230CMB, adjuvanted with either Freund's or Alhydrogel® and administered to rabbits subcutaneously or intramuscularly using a prime-boost regimen on days 0 and 28, elicited high titers of serum IgG antibodies with greater than 99 % transmission blocking activity in the presence of complement ($p < 0.001$), as determined by SMFA (Farrance et al. 2011a). These results indicate strong potential of the plant-produced Pfs230 vaccine candidate for clinical development.

7.3.3.3 Pfs25 and Pfs28

TBV candidates comprising different versions of full-length Pfs25 have also been produced in plants using the launch vector-based transient expression system. In an initial study, four soluble variants of full-length, 6xHis-tagged Pfs25 antigen containing the endoplasmic reticulum retention signal, KDEL, were produced in *N. benthamiana*: a wild-type amino acid sequence (Pfs25F1E) and a mutated version to remove two putative N-linked glycosylation sites (Pfs25MF1E), both as stand-alone proteins and as C-terminal fusions to a modified lichenase carrier protein, LicKM (Musiychuk et al. 2007), Pfs25F3E and Pfs25MF3E for the wild-type and mutated versions, respectively. These designs were engineered to determine the effects of Pfs25 fusion to LicKM, which has been shown to stabilize various targets (Musiychuk et al. 2007), as well as Pfs25 glycosylation, on the generation of functional immune responses (Farrance et al. 2011b). Although not glycosylated in *P. falciparum*, Pfs25 contains two predicted sites for N-linked glycosylation in higher eukaryotic systems. To eliminate potential N-linked glycosylation sites that had a high probability of utilization (NetNGlyc 1.0), point mutations were introduced into Pfs25 at amino acid positions 90 and 165 of the mature sequence, changing asparagine (N) to glutamine (Q) residues. For the four target antigens, expression levels ranged from 250 to 400 mg/kg of fresh leaf tissue, and solubility was approximately 100 % in extraction buffer containing 0.5 % Triton X-100. Each target, purified using a multistep purification procedure including IMAC, ion exchange chromatography, and hydrophobic interaction chromatography, contained one predominant band and had a purity of greater than 90 %, as demonstrated by SDS-PAGE followed by Coomassie staining, together with immunoblot analysis with an anti-His tag monoclonal antibody. The lack of glycosylation of the mutant Pfs25 proteins was confirmed by electrospray ionization mass spectroscopy mass determination. Also, all four Pfs25 antigens showed discrete SEC elution peaks, with expected MALLS-calculated molecular weights across the main peak (Pfs25F1E, 27,250 Da; Pfs25MF1E, 17,630 Da; Pfs25F3E, 53,310 Da; and Pfs25MF3E, 46,520 Da). Additionally, all four plant-produced Pfs25 antigens were recognized by an anti-Pfs25 transmission blocking monoclonal antibody, as determined by ELISA analysis, indicating correct protein conformation and the presence of this transmission blocking epitope in the plant-produced Pfs25 proteins.

Each variant of plant-produced Pfs25 was administered to mice as two 5 μg doses, with the antigens adsorbed onto Alhydrogel® adjuvant, to compare antigenicity and the ability to induce transmission blocking antibodies. Both fused and non-fused Pfs25 molecules induced serum anti-Pfs25 IgG antibodies with the highest titers generated by the LicKM fusion variants and the lowest titer elicited by the glycosylated non-fused variant. With all four variants, IgG1 was the predominant isotype produced, but the LicKM fusions also generated IgG2a and IgG2b.

Antibodies elicited by three of the four Pfs25 variants (both glycosylated and non-glycosylated LicKM fusion antigens and the non-fused non-glycosylated antigen) demonstrated positive staining of gametocytes and gametes in immunofluorescent assays and potent transmission blocking activity, with 97–100 % reduction in the number of malaria oocysts in SMFA, as assessed 3 weeks after the second

immunization. In contrast, transmission blocking activity of serum antibodies elicited by the non-fused glycosylated variant of Pfs25 was considerably lower (47 %). These findings suggest that N-linked glycans interfere with the ability of Pfs25 to induce a functional immune response and that fusion to LicKM compensates for this negative effect (Farrance et al. 2011b).

Since the LicKM-fused non-glycosylated variant of Pfs25 gave the highest antibody titers and complete transmission blocking, this target was selected for evaluation of the longevity of the transmission blocking immune response. The Pfs25 fusion protein (Pfs25-FhCMB) elicited antibodies with transmission blocking activity that extended out to 6 months after the second of two antigen doses (Jones et al. In Press).

Another approach to produce an efficient TBV is based on the use of VLPs. As subunit vaccines, VLPs are superior to soluble antigens. Due to their particulate structure, multivalent epitope organization, correct antigen conformation, and in some cases the presence of immunostimulatory residual host cell components, VLPs can stimulate both innate and adaptive immune responses, resulting in effective target-specific protection against pathogens (Deml et al. 2005; Grgacic and Anderson 2006; Chakerian 2007). Therefore, in an effort to produce a highly immunogenic malaria vaccine candidate with strong transmission blocking activity, a non-glycosylated version of Pfs25 (N to Q mutations as described above) was fused to the N-terminus of *Alfalfa mosaic virus* (AlMV) coat protein (CP) (Jones et al. 2013a). This Pfs25-CP VLP was transiently expressed in *N. benthamiana* plants using the launch vector system. Infiltrated plants accumulated the recombinant protein with peak levels of approximately 50 mg/kg at 7 days post-infiltration. Target VLPs were purified using polyethylene glycol precipitation followed by a three-column chromatography process, yielding >85 % purity. SDS-PAGE and immunoblot analyses using anti-Pfs25 and anti-AlMV CP antibodies demonstrated the presence of three main polypeptide chains: one at 50 kDa accounting for 20–30 % of the product and corresponding to the Pfs25-CP fusion and two others at ~25 kDa and corresponding to truncated CP versions resulting from *in planta* cleavage of the Pfs25-CP fusion polypeptide. Purified Pfs25-CP VLPs were highly consistent in size (19.3 ± 2.4 nm in diameter) with an estimated 20–30 % incorporation of Pfs25 onto the VLP surface, as shown by transmission electron microscopy and immunogold labeling. Dynamic light scattering analysis demonstrated that the particle population had a hydrodynamic radius (R_H) of 14.1 ± 1.1 nm with low polydispersity (<15 %), which was confirmed by analytical SEC. Taken together, these data indicate that Pfs25-CP VLPs represent a homogenous particle species and that the Pfs25-CP fusion and truncated CPs co-assemble *in planta* to form VLPs (Jones et al. 2013a).

Immunization of mice with Pfs25-CP VLPs adsorbed onto Alhydrogel® adjuvant, using either a two-dose or a single-dose vaccination regimen, at Pfs25 doses of 0.1–1 μg or 0.2–25 μg, respectively, induced serum antibodies predominantly of the IgG1 subclass, which exhibited complete transmission blocking activity against *P. falciparum* in SMFA that was first observed 7 weeks after the booster dose and was maintained through 6 months post-dosing. In both regimens, the adjuvant

Alhydrogel® was necessary to achieve and maintain functional antibody responses. This is the first report demonstrating complete transmission blocking after a single dose lasting at least 6 months (Jones et al. 2013a).

Pfs28 was similarly expressed in *N. benthamiana*, both alone and as fusions to carrier molecules (as a C-terminal fusion to LicKM and as an N-terminal fusion to AlMV CP), using the launch vector-based transient expression system. Although partial transmission blocking activity was observed in each case, the plant-produced Pfs28 antigens were not as efficacious as the Pfs25 antigens and were therefore not pursued further.

7.3.4 Progression of Lead Plant-Produced TBV Candidates to cGMP Production and the Clinic

The lead Pfs25-FhCMB and Pfs25-CP VLP molecules described above have been progressed through pilot-scale cGMP production, to the point of preparing these molecules for Phase 1 clinical trials. Processes for expression and recovery of each target were scaled up to the 1 kg and then 5 kg plant biomass levels. The products were purified using a three-step chromatography and a two-step ultrafiltration/diafiltration (Pfs25-FhCMB) (Jones et al. In Press) or a polyethylene glycol precipitation followed by a three-step chromatography and a two-step ultrafiltration/diafiltration (Pfs25-CP VLP) (Jones et al. 2013a) approaches. Purified vaccine candidates were characterized by SDS-PAGE, immunoblotting, reverse-phase ultra-performance liquid chromatography (RP-UPLC), SEC-MALLS, N-terminal sequencing, amino acid analysis, and peptide mapping by liquid chromatography-tandem mass spectrometry to confirm target identity and purity (>94 % for Pfs25-FhCMB and >85 % for Pfs25-CP VLP). In addition, transmission electron microscopy, dynamic light scattering analysis, and analytical SEC demonstrated that purified Pfs25-CP VLPs represent homogeneous particles of 19.3 ± 2.4 nm in diameter. For each target, in-process analytical tests allowed for monitoring of solution state and molecular weight of the target (SEC), sample purity (RP-UPLC), nickel contamination from IMAC purification of Pfs25-FhCMB (trace elemental analysis), and microbial endotoxin contamination (bioburden/agroburden tests). Both vaccine candidates showed transmission blocking activity in mice when administered with Alhydrogel® adjuvant, confirming successful scale-up to the 5 kg plant biomass level.

For manufacturing under cGMP conditions, vaccine antigen production was scaled up to the 50 kg biomass level in a pilot manufacturing facility, and characterization and in-process monitoring were performed using the criteria developed at the 5 kg biomass scale. Quality control samples were obtained for release testing and ongoing stability assessment. This release analysis included tests for target identity, strength, purity, and quality. Further assays for safety included determining the bioburden (sterility testing), bacterial endotoxin, host cell protein and DNA,

and residual levels of antifoam, kanamycin, nickel (in the case of Pfs25-FhCMB), nicotine, Triton X-100, and imidazole. Both vaccine candidates met the release criteria and demonstrated transmission blocking activity in mice when administered with Alhydrogel® adjuvant, confirming successful scale-up to the 50 kg plant biomass level under cGMP.

As an initial assessment of the technology in human volunteers, the Pfs25-CP VLP candidate has been formulated as a parenteral solution for intramuscular administration and stored in accordance with cGMP guidelines at or below −60 °C for use in a nonclinical toxicology study and immunogenicity studies. Positive results have been obtained in these nonclinical studies, and consequently, an Investigational New Drug application was submitted to the FDA in late 2013, with the vaccine candidate scheduled to progress through clinical evaluation in a Phase 1 trial (NCT02013687) in 2014 (ClinicalTrials.gov 2013b).

7.4 Criteria to Be Met to Progress Lead Plant-Produced Targets to Full Clinical Development and the Market

There are several important criteria to be met in order to advance a plant-produced TBV into advanced clinical trials and ultimately onto the market. These include demonstration of safety, immunogenicity and the induction of antibodies with transmission blocking activity in Phase 1 clinical trials, the establishment of cGMP production at commercial scale, the development of validated assays for bulk target release and formulated drug substance release, and production costs compatible with administration primarily in sub-Saharan Africa. Additional criteria that are highly preferred for an efficacious TBV include product stability at nonfrozen and preferably ambient temperatures to allow for distribution to tropical sites with limited infrastructure, the requirement for a limited number of doses to generate strong immune responses, and the maintenance of high antibody titers out to at least 6 months or preferably multiple years post-dosing to limit the need for repeat vaccinations.

As a significant step along the path of developing a TBV, Fraunhofer Center for Molecular Biotechnology has built an automated cGMP facility (Wirz et al. 2012) in Newark, DE, for the pilot-scale production of recombinant subunit vaccines, monoclonal antibodies, and other therapeutic proteins as well as protein diagnostics, reagents, and industrial enzymes (Fraunhofer Center for Molecular Biotechnology website). Quality systems have been put in place at the facility to ensure compliance with the FDA guidelines for cGMP. The quality systems act as a framework to ensure control of processes from raw materials to final product. Included in the control of product are stability programs for final product and precursors to final product. In order to show the control of drug substance, a variety of release criteria have been established. Acceptance criteria have been defined based on data from pilot batches, and drug substance is released to meet these

criteria. Acceptance criteria for the recombinant protein are designed to interrogate impurities, protein content, protein size and characteristics, and other general safety concerns such as bioburden and endotoxin content. The facility also allows for the production of Master and Working Cell Banks of the relevant recombinant *Agrobacterium* lines required for vacuum infiltration of plant material. Seed stocks of *N. benthamiana* are also generated at the Fraunhofer site. Both *Agrobacterium* cell banks and *N. benthamiana* seed stocks have acceptance criteria to be met for release and use.

The Fraunhofer Center has achieved significant progress with recombinant vaccine candidates using its launch vector-based production technology and cGMP facility. Two influenza vaccine candidates (recombinant hemagglutinin proteins from A/California/04/09 [H1N1] and A/Indonesia/05/05 [H5N1] strains of influenza virus) were produced under cGMP at pilot scale (Shoji et al. 2011) and demonstrated safety, tolerability, and immunogenicity in clinical studies (Chichester et al. 2012; Cummings et al. 2014). More recently, as described above, the center has advanced two lead TBV candidates based on Pfs25 through cGMP production at the pilot scale, and one of these candidates will complete a Phase 1 clinical trial in 2014. Promising results with this vaccine candidate should stimulate further downstream process development to improve yields to reduce product cost, as well as raise target purity to at least over 95 % to match typical purity values for recombinant subunit vaccines produced in both plant-based (Bendandi et al. 2010; Landry et al. 2010; Lai and Chen 2012; Hamorsky et al. 2013) and non-plant-based (Volkin et al. 2001; Huang et al. 2007; Wenger et al. 2007; Deschuyteneer et al. 2010; Baek et al. 2011) expression platforms. In addition, validated assays, such as a host cell protein assay for bulk target and formulated product release, will need to be developed. These advancements should lead to later stage clinical studies to address issues of necessary dosing and maintenance of transmission blocking antibody titers in vaccinated individuals. The need for improved stability during distribution emphasizes the need for extensive formulation development, with a focus on stabilization technologies such as lyophilization and spray drying.

Progressing to the market will require scale-up to commercial manufacturing scale. In the 1990s and early 2000s, there was considerable success in advancing oral plant-based vaccines into clinical trials (reviewed by Tacket 2009). The premise for these approaches was very low costs of goods for manufacturing products for oral delivery from field or greenhouse grown plants. However, these approaches raised concerns over containment and compatibility with cGMP manufacturing and formulation as well as standard approaches for storage, distribution, and administration, essentially requiring a new paradigm for human vaccine production. Therefore, it has been challenging to advance these vaccine candidates further through clinical development, and the focus has shifted to the cGMP production of high-purity recombinant proteins using plant-based expression systems where the host species is grown under tightly controlled and contained conditions. Progress observed recently in clinical development and commercialization of plant-produced pharmaceuticals manufactured using such systems has occurred due to a combination of factors, such as focusing on a small number of

production platforms; development of molecular engineering strategies to accommodate high-level expression of authentic, diverse targets; and downstream processing procedures to increase yields. This focus has allowed for reduced costs, engineering of posttranslational modifications to produce more native-like targets, development of a regulatory framework, and exploitation of niche markets to establish positions of strength for plant-made products (Fischer et al. 2013).

Several efforts are already under way to build and validate medium- to large-scale cGMP-compliant manufacturing facilities for plant-based production systems. Most notably, Protalix BioTherapeutics Inc. utilizes a novel bioreactor plant cell system, ProCellEx™, based on disposable sterile plastic bags, for manufacturing plant-derived pharmaceuticals (Protalix BioTherapeutics website). Taliglucerase alfa, produced in transgenic carrot cell culture at commercial scale under cGMP, has been approved by the FDA for the treatment of Gaucher disease (Protalix BioTherapeutics website). Also, Sigma-Aldrich Fine Chemicals (SAFC; St. Louis, MI) built animal source raw material-free cGMP facility for the downstream processing and purification of plant-based biologics (Sigma-Aldrich website).

Recently, three large-scale facilities have been constructed that focus on transiently expressing *Nicotiana* systems similar to that used by Fraunhofer Center for Molecular Biotechnology to produce the TBV candidates discussed above. Kentucky BioProcessing, LLC (KBP; Owensboro, KY), is a facility specializing in transient expression and production of recombinant proteins from *Nicotiana* plants and serves as a contract manufacturer of plant-derived pharmaceutical proteins developed by other organizations (Kentucky BioProcessing website). The FDA-approved personalized single-chain idiotype vaccines developed by Large Scale Biology Corporation for the treatment of non-Hodgkin's lymphoma (McCormick et al. 2008) were produced at KBP's cGMP facility using the TMV-based Geneware® expression technology (Kentucky BioProcessing 2008a; Fischer et al. 2012). More recently, KBP entered into an agreement with Bayer Innovation GmbH to adapt KBP's cGMP facility for use of the deconstructed plant viral vector technology MagnICON® (Gleba et al. 2005) to produce pharmaceutical proteins and other high-value products in *Nicotiana* plants at commercial scale (Kentucky BioProcessing 2008b). In addition, KBP scaled up a manufacturing process developed by PlantForm Corporation (Guelph, ON, Canada) and produced a plant-derived biosimilar version of Trastuzumab for breast cancer (PlantForm Corporation 2011). A norovirus VLP-based vaccine (noroVAXX) that was developed at Arizona State University and initially produced in its small cGMP facility (Lai and Chen 2012) was also subsequently manufactured by KBP (Fischer et al. 2012).

A second large-scale facility designed for cGMP production of plant-based pharmaceutical proteins is located in North Carolina and is run by Medicago Inc. (Medicago USA website), which was acquired by Mitsubishi Tanabe Pharma in September 2013. Medicago also operates a pilot-scale cGMP production facility in Quebec City, Canada. Medicago has been producing clinical-grade material for hemagglutinin VLP-based vaccine candidates against seasonal (A/California/04/09 [H1N1], H3N2, and B influenza strains) and pandemic (A/Indonesia/05/05 [H5N1]) influenza viruses (Medicago Inc. website) and relies on a *Cowpea mosaic virus*

hypertranslatable (CPMV-*HT*) (Sainsbury and Lomonossoff 2008) expression technology (Medicago 2009, 2011; D'Aoust et al. 2010). With support from the Defense Advanced Research Projects Agency (DARPA), Medicago USA's cGMP commercial-scale facility has been used to complete a key milestone, the production of more than ten million doses of an H1N1 VLP influenza vaccine candidate within 1 month (Medicago USA website).

Caliber Biotherapeutics, LLC, in strategic alliance with G-Con Manufacturing, operates the world's largest cGMP-compliant *N. benthamiana* expression-based protein manufacturing facility, which is located in Texas. The company is interested in "biobetter" monoclonal antibodies for the treatment of cancer. With support from DARPA, the company demonstrated rapid production of substantial quantities of pandemic influenza vaccine antigen (Caliber Biotherapeutics website).

Thus, there are large-scale facilities available that should be suitable or adaptable for the production of plant-based malaria TBV target antigens. Once an antigen has been manufactured and the bulk drug substance released, there are many commercial facilities available for formulation and fill-finish of final product for vaccine administration. Regulations and cGMP guidelines, developed by the FDA and the US Department of Agriculture (USDA), are flexible in terms of plant production platforms and apply to both stable and transient expression, as well as to both whole plants and contained plant cell culture systems (FDA/USDA 2002; Fischer et al. 2012). By contrast, current European regulatory guidelines apply only to stable transgenic plants (EMA 2008; Fischer et al. 2012), so there is a need to advance regulatory guidance here.

Cost modeling based on current expression levels achieved by Fraunhofer Center for Molecular Biotechnology with Pfs25 targets expressed in plants indicates production costs of approximately US$0.5 per dose of bulk drug substance (antigen). Since lead candidates have shown promising transmission blocking results with the established and relatively inexpensive adjuvant Alhydrogel®, this production cost is broadly compatible with target total costs for malaria vaccines of US$2 per administered dose, determined through the Malaria Vaccine Initiative's assessment of the global demand for a malaria vaccine and of the financial and social value of investing in vaccine candidates. This cost scenario assumes an incremental vaccine delivery cost of US$5 per course, with the US$ values referring to 2003 US$ (The Boston Consulting Group 2005). At a cost of US$1–2 per vaccine dose, high cost-effectiveness is predicted for most African settings even with a four-dose schedule (Moorthy et al. 2012).

7.5 Conclusions and Future Directions

In summary, TBVs offer an important strategy to interrupt the life cycle of the *Plasmodium* parasite while it is developing in the gut of the insect vector. Extensive discovery research has identified lead molecules displayed during the sexual stages of the parasite's life cycle that when injected into animals raise antibodies that can

interfere with parasite development in mosquitoes following a blood meal and thus can block the next round of transmission. Although these vaccines do not protect a vaccinated individual from contracting malaria, nor ameliorate disease symptoms, they do have the potential to limit greatly the spread of the disease and are considered important for eradicating malaria from endemic regions. Unfortunately, lead candidate antigens for TBVs have proven challenging to express in recombinant systems, and therefore, there has been interest in exploring alternative production platforms, including plants and algae. Recent reports demonstrate that both platforms have successfully been applied to express target molecules with transmission blocking activity (Farrance et al. 2011a, b; Gregory et al. 2012; Jones et al. 2013a, b), with a transiently expressing plant system particularly promising for recalcitrant antigens (Farrance et al. 2011a; Mamedov et al. 2012).

The plant-based production system that utilizes *Agrobacterium* delivery of launch vectors has been used to produce influenza subunit vaccine candidates under cGMP that have successfully completed Phase 1 clinical studies, demonstrating safety and immunogenicity (Chichester et al. 2012; Cummings et al. 2014). The same system has now been applied to produce two lead TBV candidates under cGMP in *N. benthamiana*, with one of these scheduled to complete a clinical trial in 2014. The production technology is relatively straightforward to scale, and indeed three facilities designed to operate at the metric ton plant biomass scale and compatible with this technology have been constructed. Thus, it should be clear within the next year whether plants can produce TBV candidates that are demonstrated to be safe through a Phase 1 clinical trial and capable of inducing transmission blocking antibodies in humans, and it has been shown that the technology can be ramped up for large-scale production. Extensive process development is anticipated as lead product candidates progress through later stage clinical trials to ensure that the economics of large-scale production are compatible with a cost-realistic TBV. Furthermore, success with this initial TBV candidate will likely spur efforts with other transmission blocking antigens, since a more efficacious vaccine is likely to result from including antigens expressed at different stages during parasite development, for example, P25 or P28 along with P230 or P48/45. Other target molecules are also being identified as TBV candidates (Dinglasan et al. 2007; Lavazec et al. 2007; Blagborough and Sinden 2009; Sutherland 2009; Hirai and Mori 2010; Kumar et al. 2010; Talman et al. 2011; Williams et al. 2013), and expression of these in established plant systems is anticipated. Ultimately, it is expected that a malaria TBV will be incorporated into a vaccine formulation that also includes recombinant molecules, such as CSP, that target other phases of the parasite's life cycle. Establishing a dosing regimen that most effectively confers protection and blocks parasite transmission will then be the focus of extensive later stage clinical trials.

There is considerable interest and momentum in developing effective and economically viable malaria vaccines. Indeed, the eradication of malaria is a major focus for the Bill and Melinda Gates Foundation, and the Malaria Vaccine Initiative is specifically focused on accelerating the development of malaria vaccines and their introduction into the field. Thus, developing cost-effective vaccines

to combat malaria provides an excellent opportunity for plant-based production systems to demonstrate their potential. Guiding such programs through the full extent of clinical trials will also necessitate the development of validated assays for bulk target antigen and formulated drug product release, such as ELISA-based assays for host cell protein, that will more generally benefit establishing plant-based platforms for subunit vaccine and protein therapeutic production. Success in developing a cost-effective malaria vaccine will also demonstrate the applicability of plant-based vaccines to programs to combat other parasitic diseases, which are generally cost prohibitive and fall into the category of neglected diseases.

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References

Aly ASI, Vaughan AM, Kappe SHI (2009) Malaria parasite development in the mosquito and infection of the mammalian host. Annu Rev Microbiol 63:195–221

American Society of Health-System Pharmacists [ASHSP] (2012) Pharmacy News. Taliglucerase Alfa Approved for Gaucher Disease. http://www.ashp.org/menu/News/PharmacyNews/NewsArticle.aspx?id=3718. Cited 12 Dec 2013

Baek JO, Seo JW, Kim IH, Kim CH (2011) Production and purification of human papillomavirus type 33 L1 virus-like particles from Spodoptera frugiperda 9 cells using two-step column chromatography. Protein Expr Purif 75:211–217

Barr PJ, Green KM, Gibson HL, Bathurst IC, Quakyi IA, Kaslow DC (1991) Recombinant Pfs25 protein of Plasmodium falciparum elicits malaria transmission-blocking immunity in experimental animals. J Exp Med 174:1203–1208

Bendandi M, Marillonnet S, Kandzia R, Thieme F, Nickstadt A, Herz S, Fröde R, Inogés S, Lòpez-Dìaz de Cerio A, Soria E, Villanueva H, Vancanneyt G, McCormick A, Tusé D, Lenz J, Butler-Ransohoff JE, Klimyuk V, Gleba Y (2010) Rapid, high-yield production in plants of individualized idiotype vaccines for non-Hodgkin's lymphoma. Ann Oncol 21:2420–2427

Blagborough AM, Sinden RE (2009) Plasmodium berghei HAP2 induces strong malaria transmission-blocking immunity in vivo and in vitro. Vaccine 27:5187–5194

Bousema JT, Drakeley CJ, Sauerwein RW (2006) Sexual-stage antibody responses to P. falciparum in endemic populations. Curr Mol Med 6:223–229

Caliber Biotherapeutics. http://www.caliberbio.com. Cited 12 Dec 2013

Carter R, Graves PM, Keister DB, Quakyi IA (1990) Properties of epitopes of Pfs 48/45, a target of transmission blocking monoclonal antibodies, on gametes of different isolates of Plasmodium falciparum. Parasite Immunol 12:587–603

Carter R, Coulson A, Bhatti S, Taylor BJ, Elliott JF (1995) Predicted disulfide-bonded structures for three uniquely related proteins of Plasmodium falciparum, Pfs230, Pfs48/45 and Pf12. Mol Biochem Parasitol 71:203–210

Cech PG, Aebi T, Abdallah MS, Mpina M, Machunda EB, Westerfeld N, Stoffel SA, Zurbriggen R, Pluschke G, Tanner M, Daubenberger C, Genton B, Abdulla S (2011) Virosome-formulated Plasmodium falciparum AMA-1 & CSP derived peptides as malaria vaccine: randomized phase 1b trial in semi-immune adults & children. PLoS One 6:e22273

Centers for Disease Control and Prevention [CDC] (2012) Malaria and travelers. http://www.cdc.gov/malaria/travelers/. Cited 12 Dec 2013

Chakerian B (2007) Virus-like particles: flexible platforms for vaccine development. Expert Rev Vaccines 6:381–390

Chichester JA, Musiychuk K, de la Rosa P, Horsey A, Stevenson N, Ugulava N, Rabindran S, Palmer GA, Mett V, Yusibov V (2007) Immunogenicity of a subunit vaccine against Bacillus anthracis. Vaccine 25:3111–3114

Chichester JA, Musiychuk K, Farrance CE, Mett V, Lyons J, Mett V, Yusibov V (2009) A single component two-valent LcrV-F1 vaccine protects non-human primates against pneumonic plague. Vaccine 27:3471–3474

Chichester JA, Jones RM, Green BJ, Stow M, Miao F, Moonsammy G, Streatfield SJ, Yusibov V (2012) Safety and immunogenicity of a plant-produced recombinant hemagglutinin-based influenza vaccine (HAI-05) derived from A/Indonesia/05/2005 (H5N1) influenza virus: a phase 1 randomized, double-blind, placebo-controlled, dose-escalation study in healthy adults. Viruses 4:3227–3244

Chowdhury DR, Angov E, Kariuki T, Kumar N (2009) A potent malaria transmission blocking vaccine based on codon harmonized full length Pfs48/45 expressed in Escherichia coli. PLoS One 4:e6352

ClinicalTrials.gov (2013a) Initial study of malaria vaccine Pfs25-EPA/Alhydrogel (registered trademark). http://www.clinicaltrials.gov/ct2/show/NCT01434381. Cited 12 Dec 2013

ClinicalTrials.gov (2013b) Safety and immunogenicity of plant-derived Pfs25 VLP-FhCMB malaria transmission blocking vaccine in healthy adults. http://www.clinicaltrials.gov/ct2/show/NCT02013687?term=malaria+and+plants&rank=2. Cited 11 Jan 2014

Clyde DF (1990) Immunity to falciparum and vivax malaria induced by irradiated sporozoites: a review of the University of Maryland studies, 1971-75. Bull World Health Organ 68(Suppl):9–12

Community Research and Development Information Service [CORDIS] (2012) European Commission. Periodic report summary – REDMAL (clinical development of a Pfs48/45-based malaria transmission blocking vaccine). http://cordis.europa.eu/search/index.cfm?fuseaction=result.document&RS_LANG=EN&RS_RCN=13435122&q. Cited 12 Dec 2013

Cummings JF, Guerrero ML, Moon JE, Waterman P, Nielsen RK, Jefferson S, Gross FL, Hancock K, Katz JM, Yusibov V, Fraunhofer USA Center for Molecular Biotechnology Study Group (2014) Vaccine safety and immunogenicity of a plant-produced recombinant monomer hemagglutinin-based influenza vaccine derived from influenza A (H1N1)pdm09 virus: a Phase 1 dose-escalation study in healthy adults. Vaccine 32(19):2251–2259 [Epub ahead of print]

D'Aoust MA, Couture MM, Charland N, Trépanier S, Landry N, Ors F, Vézina LP (2010) The production of hemagglutinin-based virus-like particles in plants: a rapid, efficient and safe response to pandemic influenza. Plant Biotechnol J 8:607–619

Dauvillée D, Delhaye S, Gruyer S, Slomianny C, Moretz SE, d'Hulst C, Long CA, Ball SG, Tomavo S (2010) Engineering the chloroplast targeted malarial vaccine antigens in Chlamydomonas starch granules. PLoS One 5:e15424

Deml L, Speth C, Dierich MP, Wolf H, Wagner R (2005) Recombinant HIV-1 Pr55gag virus-like particles: potent stimulators of innate and acquired immune responses. Mol Immunol 42:259–277

Deschuyteneer M, Elouahabi A, Plainchamp D, Plisnier M, Soete D, Corazza Y, Lockman L, Giannini S, Deschamps M (2010) Molecular and structural characterization of the L1 virus-like particles that are used as vaccine antigens in Cervarix™, the AS04-adjuvanted HPV-16 and -18 cervical cancer vaccine. Hum Vaccin 6:407–419

Dinglasan RR, Kalume DE, Kanzok SM, Ghosh AK, Muratova O, Pandey A, Jacobs-Lorena M (2007) Disruption of Plasmodium falciparum development by antibodies against a conserved mosquito midgut antigen. Proc Natl Acad Sci U S A 104:13461–13466

Duffy PE, Kaslow DC (1997) A novel malaria protein, Pfs28, and Pfs25 are genetically linked and synergistic as falciparum malaria transmission-blocking vaccines. Infect Immun 65:1109–1113

Enserink M (2010) Malaria's drug miracle in danger (News article). Science 138:844–846

Epstein JE, Tewari K, Lyke KE, Sim BK, Billingsley PF, Laurens MB, Gunasekera A, Chakravarty S, James ER, Sedegah M, Richman A, Velmurugan S, Reyes S, Li M,

Tucker K, Ahumada A, Ruben AJ, Li T, Stafford R, Eappen AG, Tamminga C, Bennett JW, Ockenhouse CF, Murphy JR, Komisar J, Thomas N, Loyevsky M, Birkett A, Plowe CV, Loucq C, Edelman R, Richie TL, Seder RA, Hoffman SL (2011) Live attenuated malaria vaccine designed to protect through hepatic $CD8^+$ T cell immunity. Science 334:475–480

European Medicines Agency [EMA] (2008) Guideline on the quality of biological active substances produced by stable transgene expression in higher plants. http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500003154.pdf. Cited 12 Dec 2013

Farrance CE, Rhee A, Jones RM, Musiychuk K, Shamloul M, Sharma S, Mett V, Chichester JA, Streatfield SJ, Roeffen W, van de Vegte-Bolmer M, Sauerwein RW, Tsuboi T, Muratova OV, Wu Y, Yusibov V (2011a) A plant-produced Pfs230 vaccine candidate blocks transmission of Plasmodium falciparum. Clin Vaccine Immunol 18:1351–1357

Farrance CE, Chichester JA, Musiychuk K, Shamloul M, Rhee A, Manceva SD, Jones RM, Mamedov T, Sharma S, Mett V, Streatfield SJ, Roeffen W, van de Vegte-Bolmer M, Sauerwein RW, Wu Y, Muratova O, Miller L, Duffy P, Sinden R, Yusibov V (2011b) Antibodies to plant-produced Plasmodium falciparum sexual stage protein Pfs25 exhibit transmission blocking activity. Hum Vaccin 7(Suppl):191–198

Fischer R, Schillberg S, Hellwig S, Twyman RM, Drossard J (2012) GMP issues for recombinant plant-derived pharmaceutical proteins. Biotechnol Adv 30:434–439

Fischer R, Schillberg S, Buyel JF, Twyman RM (2013) Commercial aspects of pharmaceutical protein production in plants. Curr Pharm Des 19:5471–5477

Fraunhofer USA Center for Molecular Biotechnology. http://www.fhcmb.org. Cited 12 Dec 2013

Genton B, Pluschke G, Degen L, Kammer AR, Westerfeld N, Okitsu SL, Schroller S, Vounatsou P, Mueller MM, Tanner M, Zurbriggen R (2007) A randomized placebo-controlled phase Ia malaria vaccine trial of two virosome-formulated synthetic peptides in healthy adult volunteers. PLoS One 2:e1018

Gerloff DL, Creasey A, Maslau S, Carter R (2005) Structural models for the protein family characterized by gamete surface protein Pfs230 of Plasmodium falciparum. Proc Natl Acad Sci U S A 102:13598–13603

Gleba Y, Klimyuk V, Marillonnet S (2005) Magnifection – a new platform for expressing recombinant vaccines in plants. Vaccine 23:2042–2048

Gomord V, Fitchette AC, Menu-Bouaouiche L, Saint-Jore-Dupas C, Plasson C, Michaud D, Faye L (2010) Plant-specific glycosylation patterns in the context of therapeutic protein production. Plant Biotechnol J 8:564–587

Good MF, Kaslow DC, Miller LM (1998) Pathways and strategies for developing a malaria blood-stage vaccine. Annu Rev Immunol 16:57–87

Graves PM, Wirtz RA, Carter R, Burkot TR, Looker M, Targett GA (1988) Naturally occurring antibodies to an epitope on Plasmodium falciparum gametes detected by monoclonal antibody-based competitive enzyme-linked immunosorbent assay. Infect Immun 56:2818–2821

Gregory JA, Li F, Tomosada LM, Cox CJ, Topol AB, Vinetz JM, Mayfield S (2012) Algae-produced Pfs25 elicits antibodies that inhibit malaria transmission. PLoS One 7:e37179

Gregory JA, Topol AB, Doerner DZ, Mayfield S (2013) Alga-produced cholera toxin-Pfs25 fusion proteins as oral vaccines. Appl Environ Microbiol 79:3917–3925

Grgacic EV, Anderson DA (2006) Virus-like particles: passport to immune recognition. Methods 40:60–65

Hamorsky KT, Kouokam JC, Bennett LJ, Baldauf KJ, Kajiura H, Fujiyama K, Matoba N (2013) Rapid and scalable plant-based production of a cholera toxin B subunit variant to aid in mass vaccination against cholera outbreaks. PLoS Negl Trop Dis 7:e2046

Healer J, McGuinness D, Carter R, Riley E (1999) Transmission-blocking immunity to Plasmodium falciparum in malaria-immune individuals is associated with antibodies to the gamete surface protein Pfs230. Parasitology 119(Pt 5):425–433

Hill AV (2011) Vaccines against malaria. Philos Trans R Soc Lond B Biol Sci 366:2806–2814

Hirai M, Mori T (2010) Fertilization is a novel attacking site for the transmission blocking of malaria parasites. Acta Trop 114:157–161

Hisaeda H, Stowers AW, Tsuboi T, Collins WE, Sattabongkot JS, Suwanabun N, Torii M, Kaslow DC (2000) Antibodies to malaria vaccine candidates Pvs25 and Pvs28 completely block the ability of Plasmodium vivax to infect mosquitoes. Infect Immun 68:66186623

Hoffman SL, Billingsley PF, James E, Richman A, Loyevsky M, Li T, Chakravarty S, Gunasekera A, Chattopadhyay R, Li M, Stafford R, Ahumada A, Epstein JE, Sedegah M, Reyes S, Richie TL, Lyke KE, Edelman R, Laurens MB, Plowe CV, Sim BK (2010) Development of a metabolically active, non-replicating sporozoite vaccine to prevent Plasmodium falciparum malaria. Hum Vaccin 6:97–106

Huang Y, Bi J, Zhang Y, Zhou W, Li Y, Zhao L, Su Z (2007) A highly efficient integrated chromatographic procedure for the purification of recombinant hepatitis B surface antigen from Hansenula polymorpha. Protein Expr Purif 56:301–310

Jones CS, Mayfield SP (2013) Steps toward a globally available malaria vaccine: harnessing the potential of algae for future low cost vaccines. Bioengineered 4:164–167

Jones RM, Chichester JA, Mett V, Jaje J, Tottey S, Manceva S, Casta LJ, Gibbs SK, Musiychuk K, Shamloul M, Norikane J, Mett V, Streatfield SJ, van de Vegte-Bolmer M, Roeffen W, Sauerwein RW, Yusibov V (2013a) A plant-produced Pfs25 VLP malaria vaccine candidate induces persistent transmission blocking antibodies against Plasmodium falciparum in immunized mice. PLoS One 8:e79538

Jones CS, Luong T, Hannon M, Tran M, Gregory JA, Shen Z, Briggs SP, Mayfield SP (2013b) Heterologous expression of the C-terminal antigenic domain of the malaria vaccine candidate Pfs48/45 in the green algae Chlamydomonas reinhardtii. Appl Microbiol Biotechnol 97:1987–1995

Jones RM, Chichester JA, Manceva S, Gibbs SK, Musiychuk K, Shamloul M, Norikane J, Streatfield SJ, van de Vegte-Bolmer M, Roeffen W, Sauerwein RW, Yusibov V (2014) A novel plant-produced Pfs25 fusion subunit vaccine induces long-lasting transmission blocking antibody responses. Hum Vaccin Immunother (In Press)

Kaslow DC, Quakyi IA, Syin C, Raum MG, Keister DB, Coligan JE, McCutchan TF, Miller LH (1988) A vaccine candidate from the sexual stage of human malaria that contains EGF-like domains. Nature 333:74–76

Kaslow DC, Bathurst IC, Lensen T, Ponnudurai T, Barr PJ, Keister DB (1994) Saccharomyces cerevisiae recombinant Pfs25 adsorbed to alum elicits antibodies that block transmission of Plasmodium falciparum. Infect Immun 62:5576–5580

Kentucky BioProcessing (2008a) Press release. Kentucky BioProcessing, LLC Acquires Plant Based Gene Expression Technology. http://www.kbpllc.com/News/June2008.aspx. Cited 12 Dec 2013

Kentucky BioProcessing (2008b) Press release. Bayer broadens its manufacturing basis for biopharmaceuticals in tobacco plants. http://www.kbpllc.com/News/September20083.aspx. Cited 12 Dec 2013

Kentucky BioProcessing. http://www.kbpllc.com. Cited 12 Dec 2013

Kocken CH, Jansen J, Kaan AM, Beckers PJ, Ponnudurai T, Kaslow DC, Konings RN, Schoenmakers JG (1993) Cloning and expression of the gene coding for the transmission blocking target antigen Pfs48/45 of Plasmodium falciparum. Mol Biochem Parasitol 61:59–68

Kubler-Kielb J, Majadly F, Wu Y, Narum DL, Guo C, Miller LH, Shiloach J, Robbins JB, Schneerson R (2007) Long-lasting and transmission-blocking activity of antibodies to Plasmodium falciparum elicited in mice by protein conjugates of Pfs25. Proc Natl Acad Sci U S A 104:293–298

Kumar S, Molina-Cruz A, Gupta L, Rodrigues J, Barillas-Mury C (2010) A peroxidase/dual oxidase system modulates midgut epithelial immunity in Anopheles gambiae. Science 327:1644–1648

Lai H, Chen Q (2012) Bioprocessing of plant-derived virus-like particles of Norwalk virus capsid protein under current Good Manufacture Practice regulations. Plant Cell Rep 31:573–584

Landry N, Ward BJ, Trépanier S, Montomoli E, Dargis M, Lapini G, Vézina LP (2010) Preclinical and clinical development of plant-made virus-like particle vaccine against avian H5N1 influenza. PLoS One 5:e15559

Lavazec C, Boudin C, Lacroix R, Bonnet S, Diop A, Thiberge S, Boisson B, Tahar R, Bourgouin C (2007) Carboxypeptidases B of Anopheles gambiae as targets for a Plasmodium falciparum transmission-blocking vaccine. Infect Immun 75:1635–1642

Lengeler C (2004) Insecticide-treated bed nets and curtains for preventing malaria. Cochrane Database Syst Rev (2):CD000363

Malkin EM, Durbin AP, Diemert DJ, Sattabongkot J, Wu Y, Miura K, Long CA, Lambert L, Miles AP, Wang J, Stowers A, Miller LH, Saul A (2005) Phase 1 vaccine trial of Pvs25H: a transmission blocking vaccine for Plasmodium vivax malaria. Vaccine 23:3131–3138

Mamedov T, Ghosh A, Jones RM, Mett V, Farrance CE, Musiychuk K, Horsey A, Yusibov V (2012) Production of non-glycosylated recombinant proteins in Nicotiana benthamiana plants by co-expressing bacterial PNGase F. Plant Biotechnol J 10:773–782

McCormick AA, Reddy S, Reinl SJ, Cameron TI, Czerwinkski DK, Vojdani F, Hanley KM, Garger SJ, White EL, Novak J, Barrett J, Holtz RB, Tusé D, Levy R (2008) Plant-produced idiotype vaccines for the treatment of non-Hodgkin's lymphoma: safety and immunogenicity in a phase I clinical study. Proc Natl Acad Sci U S A 105:10131–10136

Medicago Inc. (2009) Press release. PBL/John Innes Centre technology instrumental in Medicago's flu vaccine production. http://news.jic.ac.uk/2009/08/pbl-john-innes-centre-technology-instrumental-in-medicago%e2%80%99s-flu-vaccine-production/. Cited 12 Dec 2013

Medicago Inc. (2011) Press release. JIC/PBL technology aids in production of avian flu vaccine candidate. http://news.jic.ac.uk/2011/01/jic-technology-aids-production-of-avian-flu-vaccine/. Cited 12 Dec 2013

Medicago Inc. http://www.medicago.com. Cited 12 Dec 2013

Medicago USA. http://www.medicago.com/English/Medicago-USA/MedicagoUSA/default.aspx. Cited 12 Dec 2013

Mett V, Farrance CE, Green BJ, Yusibov V (2008a) Plants as biofactories. Biologicals 36:354–358

Mett V, Musiychuk K, Bi H, Farrance CE, Horsey A, Ugulava N, Shoji Y, de la Rosa P, Palmer GA, Rabindran S, Streatfield SJ, Boyers A, Russell M, Mann A, Lambkin R, Oxford JS, Schild GC, Yusibov V (2008b) A plant-produced influenza subunit vaccine protects ferrets against virus challenge. Influenza Other Respi Viruses 2:33–40

Milek RL, Roeffen WF, Kocken CH, Jansen J, Kaan AM, Eling WM, Sauerwein RW, Konings RN (1998) Immunological properties of recombinant proteins of the transmission blocking vaccine candidate, Pfs48/45, of the human malaria parasite Plasmodium falciparum produced in Escherichia coli. Parasite Immunol 20:377–385

Milek RL, Stunnenberg HG, Konings RN (2000) Assembly and expression of a synthetic gene encoding the antigen Pfs48/45 of the human malaria parasite Plasmodium falciparum in yeast. Vaccine 18:1402–1411

Miura K, Keister DB, Muratova OV, Sattabongkot J, Long CA, Saul A (2007) Transmission-blocking activity induced by malaria vaccine candidates Pfs25/Pvs25 is a direct and predictable function of antibody titer. Malar J 6:107

Moorthy VS, Hutubessy R, Newman RD, Hombach J (2012) Decision-making on malaria vaccine introduction: the role of cost-effectiveness analyses. Bull World Health Organ 90:864–866, http://www.who.int/bulletin/volumes/90/11/12-107482/en/index.html. Cited 12 Dec 2013

Musiychuk K, Stephenson N, Bi H, Farrance CE, Orozovic G, Brodelius M, Brodelius P, Horsey A, Ugulava N, Shamloul AM, Mett V, Rabindran S, Streatfield SJ, Yusibov V (2007) A launch vector for the production of vaccine antigens in plants. Influenza Other Respi Viruses 1:19–25

Nabi SA, Qader SS (2009) Is Global Warming likely to cause an increased incidence of Malaria? Libyan J Med 4:18–22

Nugent JM, Joyce SM (2005) Producing human therapeutic proteins in plastids. Curr Pharm Des 11:2459–2470

Okitsu SL, Silvie O, Westerfeld N, Curcic M, Kammer AR, Mueller MS, Sauerwein RW, Robinson JA, Genton B, Mazier D, Zurbriggen R, Pluschke G (2007) A virosomal malaria peptide vaccine elicits a long-lasting sporozoite-inhibitory antibody response in a phase 1a clinical trial. PLoS One 2:e1278

Olotu A, Fegan G, Wambua J, Nyangweso G, Awuondo KO, Leach A, Lievens M, Leboulleux D, Njuguna P, Peshu N, Marsh K, Bejon P (2013) Four-year efficacy of RTS, S/AS01E and its interaction with malaria exposure. N Engl J Med 368:1111–1120

Ouédraogo AL, Roeffen W, Luty AJ, de Vlas SJ, Nebie I, Ilboudo-Sanogo E, Cuzin-Ouattara N, Teleen K, Tiono AB, Sirima SB, Verhave JP, Bousema T, Sauerwein R (2011) Naturally acquired immune responses to Plasmodium falciparum sexual stage antigens Pfs48/45 and Pfs230 in an area of seasonal transmission. Infect Immun 79:4957–4964

Outchkourov N, Vermunt A, Jansen J, Kaan A, Roeffen W, Teelen K, Lasonder E, Braks A, van de Vegte-Bolmer M, Qiu LY, Sauerwein R, Stunnenberg HG (2007) Epitope analysis of the malaria surface antigen pfs48/45 identifies a subdomain that elicits transmission blocking antibodies. J Biol Chem 282:17148–17156

Outchkourov NS, Roeffen W, Kaan A, Jansen J, Luty A, Schuiffel D, van Gemert GJ, van de Vegte-Bolmer M, Sauerwein RW, Stunnenberg HG (2008) Correctly folded Pfs48/45 protein of Plasmodium falciparum elicits malaria transmission-blocking immunity in mice. Proc Natl Acad Sci U S A 105:4301–4305

PATH Malaria Vaccine Initiative [MVI] (2013) Press release. Malaria vaccine candidate reduces disease over 18 months of follow-up in late-stage study of more than 15,000 infants and young children. http://www.malariavaccine.org/files/RTSS_18_months_followup_RELEASE_EMBARGOED_UNTIL_0001_BST_08_Oct_2013_.pdf. Cited 12 Dec 2013

PlantForm Corporation (2011) Quarterly update. http://plantformcorp.com/newsletters/newsletter_april_2011.html. Cited 12 Dec 2013

Plummer TH Jr, Elder JH, Alexander S, Phelan AW, Tarentino AL (1984) Demonstration of peptide:N-glycosidase F activity in endo-beta-N-acetylglucosaminidase F preparations. J Biol Chem 259:10700–10704

Pradel G (2007) Proteins of the malaria parasite sexual stages: expression, function and potential for transmission blocking strategies. Parasitology 134:1911–1929

Protalix BioTherapeutics Inc. http://www.protalix.com/procellex-platform/overview-procellex-platform.asp. Cited 12 Dec 2013

Qian F, Wu Y, Muratova O, Zhou H, Dobrescu G, Duggan P, Lynn L, Song G, Zhang Y, Reiter K, MacDonald N, Narum DL, Long CA, Miller LH, Saul A, Mullen GE (2007) Conjugating recombinant proteins to Pseudomonas aeruginosa ExoProtein A: a strategy for enhancing immunogenicity of malaria vaccine candidates. Vaccine 25:3923–3933

Qian F, Rausch KM, Muratova O, Zhou H, Song G, Diouf A, Lambert L, Narum DL, Wu Y, Saul A, Miller LH, Long CA, Mullen GE (2008) Addition of CpG ODN to recombinant Pseudomonas aeruginosa ExoProtein A conjugates of AMA1 and Pfs25 greatly increases the number of responders. Vaccine 26:2521–2527

Qian F, Aebig JA, Reiter K, Barnafo E, Zhang Y, Shimp RL Jr, Rausch KM, Jones DS, Zhu D, Lambert L, Mullen GE, Narum DL, Miller LH, Wu Y (2009) Enhanced antibody responses to Plasmodium falciparum Pfs28 induced in mice by conjugation to ExoProtein A of Pseudomonas aeruginosa with an improved procedure. Microbes Infect 11:408–412

Quakyi IA, Carter R, Rener J, Kumar N, Good MF, Miller LH (1987) The 230-kDa gamete surface protein of Plasmodium falciparum is also a target for transmission-blocking antibodies. J Immunol 139:4213–4217

Rasala BA, Mayfield SP (2011) The microalga Chlamydomonas reinhardtii as a platform for the production of human protein therapeutics. Bioeng Bugs 2:50–54

Richardson JS, Richardson DC (2002) Natural β-sheet proteins use negative design to avoid edge-to-edge aggregation. Proc Natl Acad Sci U S A 99:2754–2759

Rieckmann KH (1990) Human immunization with attenuated sporozoites. Bull World Health Organ 68:13–16

Roll Back Malaria (2013) Key malaria facts. http://www.rollbackmalaria.org/keyfacts.html. Cited 12 Dec 2013

Rosenberg JN, Oyler GA, Wilkinson L, Betenbaugh MJ (2008) A green light for engineered algae: redirecting metabolism to fuel a biotechnology revolution. Curr Opin Biotechnol 19:430–436

Rybicki EP (2010) Plant-made vaccines for humans and animals. Plant Biotechnol J 8:620–637

Sainsbury F, Lomonossoff GP (2008) Extremely high-level and rapid transient protein production in plants without the use of viral replication. Plant Physiol 148:1212–1218

Samuelson J, Banerjee S, Magnelli P, Cui J, Kelleher DJ, Gilmore R, Robbins PW (2005) The diversity of dolichol-linked precursors to Asn-linked glycans likely results from secondary loss of sets of glycosyltransferases. Proc Natl Acad Sci U S A 102:1548–1553

Schwartz L, Brown GV, Genton B, Moorthy VS (2012) A review of malaria vaccine clinical projects based on the WHO rainbow table. Malar J 11:11

Seder RA, Chang LJ, Enama ME, Zephir KL, Sarwar UN, Gordon IJ, Holman LA, James ER, Billingsley PF, Gunasekera A, Richman A, Chakravarty S, Manoj A, Velmurugan S, Li M, Ruben AJ, Li T, Eappen AG, Stafford RE, Plummer SH, Hendel CS, Novik L, Costner PJ, Mendoza FH, Saunders JG, Nason MC, Richardson JH, Murphy J, Davidson SA, Richie TL, Sedegah M, Sutamihardja A, Fahle GA, Lyke KE, Laurens MB, Roederer M, Tewari K, Epstein JE, Sim BK, Ledgerwood JE, Graham BS, Hoffman SL, VRC 312 Study Team (2013) Protection against malaria by intravenous immunization with a nonreplicating sporozoite vaccine. Science 341:1359–1365

Shaaltiel Y, Bartfeld D, Hashmueli S, Baum G, Brill-Almon E, Galili G, Dym O, Boldin-Adamsky SA, Silman I, Sussman JL, Futerman AH, Aviezer D (2007) Production of glucocerebrosidase with terminal mannose glycans for enzyme replacement therapy of Gaucher's disease using a plant cell system. Plant Biotechnol J 5:579–590

Sheehy SH, Duncan CJ, Elias SC, Collins KA, Ewer KJ, Spencer AJ, Williams AR, Halstead FD, Moretz SE, Miura K, Epp C, Dicks MD, Poulton ID, Lawrie AM, Berrie E, Moyle S, Long CA, Colloca S, Cortese R, Gilbert SC, Nicosia A, Hill AV, Draper SJ (2011) Phase Ia clinical evaluation of the Plasmodium falciparum blood-stage antigen MSP1 in ChAd63 and MVA vaccine vectors. Mol Ther 19:2269–2276

Sheehy SH, Duncan CJ, Elias SC, Biswas S, Collins KA, O'Hara GA, Halstead FD, Ewer KJ, Mahungu T, Spencer AJ, Miura K, Poulton ID, Dicks MD, Edwards NJ, Berrie E, Moyle S, Colloca S, Cortese R, Gantlett K, Long CA, Lawrie AM, Gilbert SC, Doherty T, Nicosia A, Hill AV, Draper SJ (2012) Phase Ia clinical evaluation of the safety and immunogenicity of the Plasmodium falciparum blood-stage antigen AMA1 in ChAd63 and MVA vaccine vectors. PLoS One 7:e31208

Shimp RL Jr, Rowe C, Reiter K, Chen B, Nguyen V, Aebig J, Rausch KM, Kumar K, Wu Y, Jin AJ, Jones DS, Narum DL (2013) Development of a Pfs25-EPA malaria transmission blocking vaccine as a chemically conjugated nanoparticle. Vaccine 31:2954–2962

Shoji Y, Chichester JA, Bi H, Musiychuk K, de la Rosa P, Goldschmidt L, Horsey A, Ugulava N, Palmer GA, Mett V, Yusibov V (2008) Plant-expressed HA as a seasonal influenza vaccine candidate. Vaccine 26:2930–2934

Shoji Y, Bi H, Musiychuk K, Rhee A, Horsey A, Roy G, Green B, Shamloul M, Farrance CE, Taggart B, Mytle N, Ugulava N, Rabindran S, Mett V, Chichester JA, Yusibov V (2009a) Plant-derived hemagglutinin protects ferrets against challenge infection with the A/Indonesia/05/05 strain of avian influenza. Vaccine 27:1087–1092

Shoji Y, Farrance CE, Bi H, Shamloul M, Green B, Manceva S, Rhee A, Ugulava N, Roy G, Musiychuk K, Chichester JA, Mett V, Yusibov V (2009b) Immunogenicity of hemagglutinin from A/Bar-headed Goose/Qinghai/1A/05 and A/Anhui/1/05 strains of H5N1 influenza viruses produced in Nicotiana benthamiana plants. Vaccine 27:3467–3470

Shoji Y, Chichester JA, Jones M, Manceva SD, Damon E, Mett V, Musiychuk K, Bi H, Farrance C, Shamloul M, Kushnir N, Sharma S, Yusibov V (2011) Plant-based rapid production of recombinant subunit hemagglutinin vaccines targeting H1N1 and H5N1 influenza. Hum Vaccin 7(Suppl):41–50

Sigma-Aldrich. http://www.sigmaaldrich.com/safc/products/contract-manufacturing-solutions/therapeutic-proteins/native-sourced-extraction-and-purification.html. Cited 12 Dec 2013

Stoute JA, Slaoui M, Heppner DG, Momin P, Kester KE, Desmons P, Wellde BT, Garçon N, Krzych U, Marchand M (1997) A preliminary evaluation of a recombinant circumsporozoite protein vaccine against Plasmodium falciparum malaria. RTS, S Malaria Vaccine Evaluation Group. N Engl J Med 336:86–91

Sutherland CJ (2009) Surface antigens of Plasmodium falciparum gametocytes–a new class of transmission-blocking vaccine targets? Mol Biochem Parasitol 166:93–98

Tachibana M, Wu Y, Iriko H, Muratova O, MacDonald NJ, Sattabongkot J, Takeo S, Otsuki H, Torii M, Tsuboi T (2011) N-terminal prodomain of Pfs230 synthesized using a cell-free system is sufficient to induce complement-dependent malaria transmission-blocking activity. Clin Vaccine Immunol 18:1343–1350

Tachibana M, Sato C, Otsuki H, Sattabongkot J, Kaneko O, Torii M, Tsuboi T (2012) Plasmodium vivax gametocyte protein Pvs230 is a transmission-blocking vaccine candidate. Vaccine 30:1807–1812

Tacket CO (2009) Plant-based oral vaccines: results of human trials. Curr Top Microbiol Immunol 332:103–117

Talaat KR et al Phase I trial of Pfs25-EPA/Alhydrogel®: a transmission-blocking vaccine against falciparum malaria in healthy malaria-naïve adults. Presented at: ASTMH 61st annual meeting, Washington, DC, 15 Nov 2012

Talman AM, Lacroix C, Marques SR, Blagborough AM, Carzaniga R, Ménard R, Sinden RE (2011) PbGEST mediates malaria transmission to both mosquito and vertebrate host. Mol Microbiol 82:462–474

Tarentino AL, Quinones G, Trumble A, Changchien LM, Duceman B, Maley F, Plummer TH Jr (1990) Molecular cloning and amino acid sequence of peptide-N4-(N-acetyl-beta-D-glucosaminyl)asparagine amidase from flavobacterium meningosepticum. J Biol Chem 265:6961–6966

The Boston Consulting Group (2005) Market assessment for malaria vaccines. http://www.malariavaccine.org/files/MVIMarketAssessment-Shortversion-16Feb05-LB.pdf. Cited 12 Dec 2013

The RTS,S Clinical Trials Partnership (2011) First results of phase 3 trial of RTS, S/AS01 malaria vaccine in African children. N Engl J Med 365:1863–1875

The RTS,S Clinical Trials Partnership (2012) A phase 3 trial of RTS, S/AS01 malaria vaccine in African infants. N Engl J Med 367:2284–2295

Thera MA, Plowe CV (2012) Vaccines for malaria: how close are we? Annu Rev Med 63:345–357

Thera MA, Doumbo OK, Coulibaly D, Laurens MB, Kone AK, Guindo AB, Traore K, Sissoko M, Diallo DA, Diarra I, Kouriba B, Daou M, Dolo A, Baby M, Sissoko MS, Sagara I, Niangaly A, Traore I, Olotu A, Godeaux O, Leach A, Dubois MC, Ballou WR, Cohen J, Thompson D, Dube T, Soisson L, Diggs CL, Takala SL, Lyke KE, House B, Lanar DE, Dutta S, Heppner DG, Plowe CV (2010) Safety and immunogenicity of an AMA1 malaria vaccine in Malian children: results of a phase 1 randomized controlled trial. PLoS One 5:e9041

Thera MA, Doumbo OK, Coulibaly D, Laurens MB, Ouattara A, Kone AK, Guindo AB, Traore K, Traore I, Kouriba B, Diallo DA, Diarra I, Daou M, Dolo A, Tolo Y, Sissoko MS, Niangaly A, Sissoko M, Takala-Harrison S, Lyke KE, Wu Y, Blackwelder WC, Godeaux O, Vekemans J, Dubois MC, Ballou WR, Cohen J, Thompson D, Dube T, Soisson L, Diggs CL, House B, Lanar DE, Dutta S, Heppner DG Jr, Plowe CV (2011) A field trial to assess a blood-stage malaria vaccine. N Engl J Med 365:1004–1013

Tomas AM, Margos G, Dimopoulos G, van Lin LH, de Koning-Ward TF, Sinha R, Lupetti P, Beetsma AL, Rodriguez MC, Karras M, Hager A, Mendoza J, Butcher GA, Kafatos F, Janse CJ, Waters AP, Sinden RE (2001) P25 and P28 proteins of the malaria ookinete surface have multiple and partially redundant functions. EMBO J 20:3975–3983

Tran M, Zhou B, Pettersson PL, Gonzalez MJ, Mayfield SP (2009) Synthesis and assembly of a full-length human monoclonal antibody in algal chloroplasts. Biotechnol Bioeng 104:663–673

Tsuboi T, Takeo S, Iriko H, Jin L, Tsuchimochi M, Matsuda S, Han ET, Otsuki H, Kaneko O, Sattabongkot J, Udomsangpetch R, Sawasaki T, Torii M, Endo Y (2008) Wheat germ cell-free system-based production of malaria proteins for discovery of novel vaccine candidates. Infect Immun 76:1702–1708

U.S. Food and Drug Administration [FDA]/U.S. Department of Agriculture [USDA] (2002) Guidance for industry drugs, biologics, and medical devices derived from bioengineered plants for use in humans and animals. Draft guidance. http://www.fda.gov/downloads/AnimalVeterinary/GuidanceComplianceEnforcement/GuidanceforIndustry/ucm055424.pdf. Cited 12 Dec 2013

Ugwu CU, Aoyagi H, Uchiyama H (2008) Photobioreactors for mass cultivation of algae. Bioresour Technol 99:4021–4028

van Dijk MR, Janse CJ, Thompson J, Waters AP, Braks JA, Dodemont HJ, Stunnenberg HG, van Gemert GJ, Sauerwein RW, Eling W (2001) A central role for P48/45 in malaria parasite male gamete fertility. Cell 104:153–164

Vermeulen AN, Ponnudurai T, Beckers PJ, Verhave JP, Smits MA, Meuwissen JH (1985) Sequential expression of antigens on sexual stages of Plasmodium falciparum accessible to transmission-blocking antibodies in the mosquito. J Exp Med 162:1460–1476

Volkin DB, Shi L, Mach H (2001) Human papillomavirus vaccine formulations. U.S. Patent. http://www.google.com/patents?hl=en&lr=&vid=USPAT6251678&id=YisIAAAAEBAJ&oi=fnd&dq=Volkin+et+al.+2001+purity+HPV+Patent&printsec=abstract#v=onepage&q&f=false. Cited 12 Dec 2013

Wenger MD, Dephillips P, Price CE, Bracewell DG (2007) An automated microscale chromatographic purification of virus-like particles as a strategy for process development. Biotechnol Appl Biochem 47(Pt 2):131–139

Williams AR, Zakutansky SE, Miura K, Dicks MD, Churcher TS, Jewell KE, Vaughan AM, Turner AV, Kapulu MC, Michel K, Long CA, Sinden RE, Hill AV, Draper SJ, Biswas S (2013) Immunisation against a serine protease inhibitor reduces intensity of Plasmodium berghei infection in mosquitoes. Int J Parasitol 43:869–874

Williamson KC, Keister DB, Muratova O, Kaslow DC (1995) Recombinant Pfs230, a Plasmodium falciparum gametocyte protein, induces antisera that reduce the infectivity of Plasmodium falciparum to mosquitoes. Mol Biochem Parasitol 75:33–42

Wirz H, Sauer-Budge AF, Briggs J, Sharpe A, Shu S, Sharon A (2012) Automated production of plant-based vaccines and pharmaceuticals. J Lab Autom 17:449–457

World Health Organization [WHO] (2000) Malaria transmission blocking vaccines: an ideal public good. http://www.who.int/tdr/publications/documents/malaria-transmission-blocking.pdf. Cited 12 Dec 2013

World Health Organisation [WHO] (2010) Guidelines for the treatment of malaria, 2d edn. http://whqlibdoc.who.int/publications/2010/9789241547925_eng.pdf. Cited 12 Dec 2013

World Health Organisation [WHO] (2012) World malaria report. http://www.who.int/malaria/publications/world_malaria_report_2012/wmr2012_factsheet.pdf. Cited 12 Dec 2013

World Health Organization [WHO] (2013) Initiative for Vaccine Research (IVR). Tables of malaria vaccine projects globally. http://www.who.int/vaccine_research/links/Rainbow/en/. Cited 12 Dec 2013

Wu Y, Ellis RD, Shaffer D, Fontes E, Malkin EM, Mahanty S, Fay MP, Narum D, Rausch K, Miles AP, Aebig J, Orcutt A, Muratova O, Song G, Lambert L, Zhu D, Miura K, Long C, Saul A, Miller LH, Durbin AP (2008) Phase 1 trial of malaria transmission blocking vaccine candidates Pfs25 and Pvs25 formulated with montanide ISA 51. PLoS One 3:e2636

Yusibov V, Streatfield SJ, Kushnir N (2011) Clinical development of plant-produced recombinant pharmaceuticals: vaccines, antibodies and beyond. Hum Vaccin 7:313–321

Yusibov V, Streatfield SJ, Kushnir N, Roy G, Padmanaban A (2013) Hybrid viral vectors for vaccine and antibody production in plants. Curr Pharm Des 19:5574–5586

Zou L, Miles AP, Wang J, Stowers AW (2003) Expression of malaria transmission-blocking vaccine antigen Pfs25 in Pichia pastoris for use in human clinical trials. Vaccine 21:1650–1657

Chapter 8
An Oral Vaccine for TGEV Immunization of Pigs

V. Rajan

8.1 Introduction

8.1.1 Structure of TGEV

Transmissible gastroenteritis virus (TGEV) is an economically important porcine pathogen that causes severe, contagious diarrhea and vomiting with high mortality in piglets under 2 weeks of age. TGEV can manifest endemically or epidemically in swine, and the virus may be vectored in by other animals such as dogs, cats, birds, and rodents. TGEV's prevalence and detrimental effects on commercial hog farms have spurred research into vaccines, particularly those suitable for convenient administration to large numbers of pigs. Large naïve droves are costly and inconvenient to immunize with current vaccines because each individual has to be isolated, vaccinated, and tagged. Inducing oral immunity through colostrum by vaccinating sows, or boosting immunity in piglets following single primary injection, will also be beneficial due to lower associated costs.

TGEV belongs to the subfamily *Coronavirinae* in the *Coronaviridae* family of enveloped viruses (Belouzard et al. 2012). Coronaviruses cause respiratory or enteric disease in avian, bovine, feline, canine, murine, and human hosts. The most widely known virus in this class is responsible for severe acute respiratory syndrome (SARS) (Nuttall and Dye 2013), and more recently, NCoV, a novel coronavirus isolated from the Arabian Peninsula (Buchholz et al. 2013; Hofer 2013). Structurally, coronaviruses are among the largest viruses, at about 100 nm in diameter, and have a large, positive-strand RNA genome. TGEV is related to other swine coronaviruses: the porcine respiratory coronavirus (PCRV), porcine

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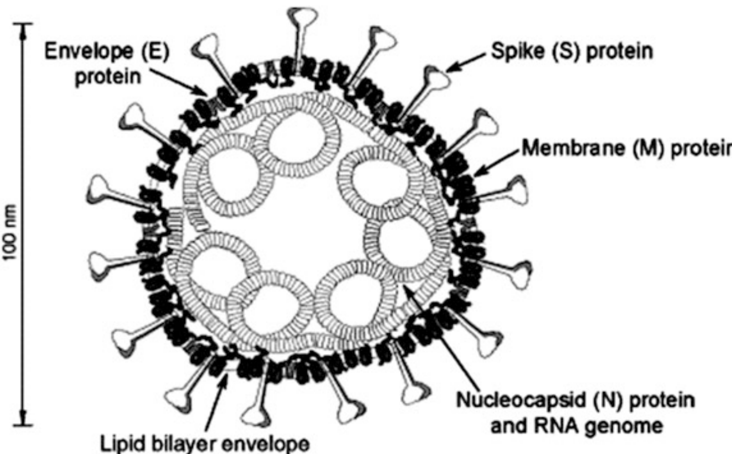

Fig. 8.1 Schematic of the coronavirus virion, with the minimal set of structural proteins. Reproduced with permission from Masters (2006)

epidemic diarrhea virus (PEDV), and porcine hemagglutinating encephalomyelitis virus (HEV) (Sestak and Saif 2008).

It is pertinent to examine the structure of TGEV and related viruses to explain the choices of epitopes available for the production of effective subunit vaccines. All coronaviruses have an envelope with radiating structures composed of trimers of the 220 kDa spike glycoprotein (S, also referred to as peplomer E2), as well as the smaller membrane glycoprotein (M, 29–36 kDa) and envelope protein (E1, 10 kDa) (Fig. 8.1). The M protein interacts with the nucleocapsid N protein and the viral RNA to form the icosahedral nucleoprotein core (Masters 2006). The S-protein, specifically the N-terminal domain between amino acids 522–744, binds aminopeptidase N receptor in the epithelium of the small intestine and mediates the fusion of the host and viral membranes and uptake (Belouzard et al. 2012).

8.1.2 *Antigenicity of TGEV's Structural Components*

The surface and subviral structural components of the virus have been assessed for antigenicity, and neutralizing antibodies were found to be associated with the surface components which function in recognition and binding with subviral and host proteins (Garwes et al. 1979). The M protein induces interferon production and also binds neutralizing antibodies (Laude et al. 1992). The S (or E2) protein has been found to be the most effective epitope at inducing neutralizing antibodies. The S-protein has four major antigenic sites at the N-terminal: A, B, C, and D; A and D are involved in antigen neutralization (Reguera et al. 2011; Correa et al. 1988;

Jiménez et al. 1986). Induction of cross-protection with the use of a related virus has been attempted. PCRV shows tropism towards respiratory tissues and differs from TGEV in having a deletion of the N-terminal 224–227 amino acids (containing antigenic sites A and D) of the S-protein, indicating that the A and D sites may be involved in tissue specificity. Immunization with PCRV was partially protective under the same conditions—33 % of piglets survived challenge (De Diego et al. 1994). The S-antigen was therefore selected as the most efficient immunogen for a subunit vaccine.

8.2 Description of the System Used to Produce the Antigen

8.2.1 Theoretical Advantages of the Maize Process Over Other Technologies

High-level expression of heterologous eukaryotic proteins has been successfully attempted in both prokaryotes and eukaryotes. However, many eukaryotic proteins require posttranslational modifications such as glycosylation, which are not carried out by prokaryotes, and so eukaryotic systems are sometimes necessary to obtain functional end products.

The benefits of plants as production systems are primarily the low cost of production and rapid scale-up that make it more responsive than other methods to high production targets. Traits introduced by transgenic techniques into separate lines can be combined by traditional breeding methods to produce lines with a combination of these traits. The production of bulk plant material requires only space, fertile soil, and sunlight, which are less onerous to provide than sterile fermentation chambers and animal care. Plants do not harbor animal pathogens, and the concern for contamination of products with animal pathogens, such as prions or viruses, is alleviated. Plant material has been shown to serve to bioencapsulate proteins, permitting slow release in the gut (Kong et al. 2001; Verma et al. 2010; Bailey 2000). Thus, there may be a degree of dosage flexibility with oral antigens administered in plant tissue, and the feed can be administered over a larger window to accommodate time frames for induction of secretory and humoral immunoglobulins (Bailey 2000; Daniell et al. 2001).

Important considerations before attempting expression of heterologous eukaryotic proteins in plants are the requirements for posttranslational protein modifications, such as glycosylation, and the presence of allergens and toxins. Glycosylation moieties and patterns differ between animals and plants which has raised concerns over the potential for nonfunctional proteins or an allergic reaction to the plant glycosylated protein. These differences, however, do not appear to be significant functionally (Ma et al. 2003; Suzuki et al. 2003), and plant carbohydrate-specific IgEs in allergic patients were shown to be clinically irrelevant when the protein is orally administered (Mari et al. 2008; Bosch and Schots 2010). This lack of allergenicity

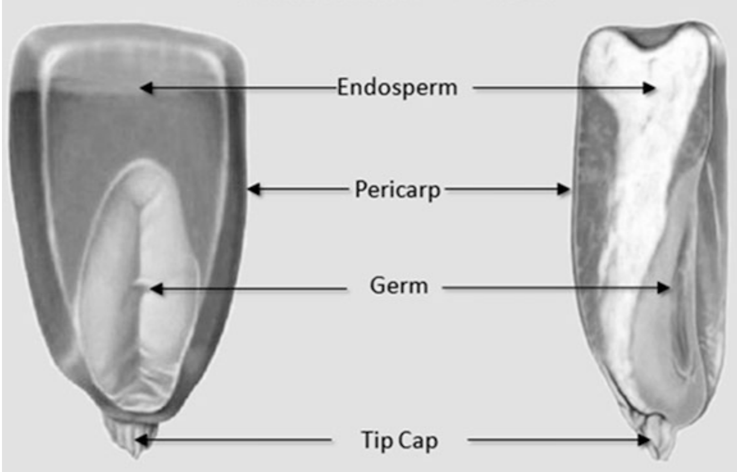

Fig. 8.2 Cross section of maize seed. Reproduced with permission from Cereal Process Technologies, LLC

may be partly explained by tolerance to plant glycosylation induced by oral exposure (see Ghaderi et al. 2012 for an overview of glycosylation of biotherapeutic proteins). Potential concerns about glycosylation-induced allergenicity may also be overcome by the removal of plant glycosylation sites, inactivation of plant glycosylases, and the expression of plant deglycosylases and mammalian-type glycosylases (Sethuraman and Stadheim 2006; Desai et al. 2010; Mamedov et al. 2012). Host plants should also be screened for toxic or allergenic metabolites that may co-purify or be co-administered with the target protein.

Edible tissues such as grain from plants with generally recognized as safe (GRAS) status may be directly used for feed-based vaccines (Naqvi et al. 2011). Seeds are a natural, desiccated, storage system lacking proteases, and recombinant proteins produced in seed have been stable at room temperature for extended periods of time (Naqvi et al. 2011). Selection and backcrossing into parent seed and hybrid lines can establish uniform concentrations of antigen expression (Hood et al. 2012) and standard processing and formulation methods can reliably produce consistent concentrations of antigens for administration, which is not straightforward with perishable edible tissues such as lettuce leaves and bananas. Bioencapsulation of the antigen in plant tissue prevents premature degradation resulting in the antigen persisting in the intestine, a key factor for the induction of a protective sIgA response, important for protection against ingested pathogens. A system that exemplifies the GRAS system is maize (or corn) grain which was used in the work described here. Its structure and specific advantages are described below.

The maize kernel contains three main regions: the embryo, the endosperm, and the pericarp (Fig. 8.2). The pericarp includes the seed coat and fruit layers. The pericarp originates from the ovary wall and functions to protect the seed. The

aleurone layer is the outermost layer of the endosperm and lies directly below the pericarp. Aleurone grains contain enzymes involved in the breakdown of starch and proteins in the germinating seed. The embryo and endosperm are well suited to heterologous protein accumulation as both are rich in protein and have associated tissue-specific promoters. The volume of the endosperm is greater than that of the embryo allowing, in theory, more protein accumulation but, in practice, embryo-preferred promoters have been used to produce some of the highest recorded concentrations of heterologous proteins (Hood et al. 2012; Egelkrout et al. 2013). Desiccation of the seed provides an environment that protects proteins from enzymatic degradation (Ma et al. 2003), and maize seed has cystatins (Yamada et al. 2000; Massonneau et al. 2005) and other protease inhibitors (Jongsma and Bolter 1997) as a defense against proteolysis by insect pathogens.

8.2.2 Theoretical Considerations for an Optimal Vaccine for Piglets

The ideal vaccine for TGEV will provide mucosal, lactogenic, and systemic immunity, while being simple to administer to droves which may number in the many thousands. Potentially, the methods to protect naïve piglets at highest risk from TGEV infection are to provide immune colostrum by vaccinating sows and an oral/nasal vaccine to boost secretory neutralizing antibody levels. Since TGEV is an enteric disease, the induction of sIgA is inferred to be more protective. It has been observed that the mucosal immunoglobulin, IgA, is more efficiently induced through gut-associated lymphoid tissue (GALT) if the antigen persists in the intestine (Foss and Murtaugh 2000). Induction of passive immunity transmitted through colostrum can be mediated by either of the soluble immunoglobulins IgG or IgA. Initially, IgG is more abundant and protects against systemic infection, whereas IgAs in colostrum provide protection to the gut lumen where the enterocytes in which TGEV replicates reside. IgA production and mucosal immunity is thus the response desired. There have been three main routes taken to effect protection in young piglets: (1) parenteral immunization with inactivated virus, (2) oral/nasal administration of attenuated virus, and (3) oral administration of viral subunit antigens in feed.

Parenteral immunization of young piglets with inactivated virus is ineffective as it does not induce the local immune response in the gut, nor are piglets at this age capable of mounting an immune response. However, parenteral immunization of pregnant sows can induce production of protective immunoglobulins in colostrum and milk. The disadvantage of this approach is the necessity of individually vaccinating sows, which can be time-consuming and expensive. Immunization must also be done on a schedule prior to birth that maximizes immunoglobulin presence in the colostrum, and that can be difficult, especially with large numbers. Nasal administration of attenuated viruses does overcome this difficulty, but each

pregnant animal still needs to be isolated and the vaccine delivered separately. As with parenteral injection, the vaccination schedule is important because, while the attenuated virus does replicate in the gut, thus stimulating antibody production over an extended period, viral replication is suboptimal. While the oral vaccine can be more easily administered, the frequency of inoculation has to be modulated to maximize colostrum immunoglobulin timed with birth.

The option of using the oral route to deliver viral subunit antigens in feed is fairly straightforward in terms of administration: the food is put out in feeding troughs and animals are monitored by observation to ensure all animals have had access to the food. However, dosage for orally administered vaccines in feed is particularly significant, because antigens could be ingested at greater or lesser doses depending on the amount of feed consumed, and some of the antigens would be expected to be destroyed by the digestive process. The typical vaccine dosage in feed may be up to 100 times greater than that used for parenteral administration, but this may be reduced by the use of suitable targeting or carrier molecules (Carter and Langridge 2002). Still, common consensus is that a precise dose, taking into account degradation in the gut, must be given to all animals. Interestingly, Verma et al. (2010) have shown that the response from a 20-fold range of antigen levels given encapsulated in plant materials is comparable.

Finally, with any vaccine, oral or parenteral, there is a concern over the potential induction of tolerance, which has been shown to occur for orally administered antigens with repeated doses of antigens over a long term and is mediated by regulatory T-cells. A single large dose has also been shown to induce anergy—a mechanism of adaptive tolerance that cause T-cells, in an environment presumably low in co-stimulators, to become unresponsive to antigens (Weiner 1997; Weiner et al. 2011). Therefore, prior to widespread vaccination, tests must be conducted to ensure this is not an issue. With these caveats addressed, plants have proven to be efficient hosts for heterologous protein production.

8.2.3 Review of Past Efforts to Produce TGEV Vaccines

De Diego et al. (1994) showed that oral immunization with TGEV was more effective at inducing the presence of neutralizing antibodies in colostrum and milk compared to intranasal immunization with PCRV. They attributed this difference to GALT being more effective than bronchus-associated lymphoid tissue (BALT), going even to the extent of questioning if BALT was indeed an integral mammalian structure (Pabst 1992). Pigs have been shown to not have BALT constitutively (Delventhal et al. 1992; Pabst and Gehrke 1990). Thus, the use of intranasal stimulation by PCRV may have been less effective for this reason. Peyer's patches in the gut have long been known to be associated with GALT, and oral immunization has been shown to be effective in producing IgA and IgG globulins in milk and was 100 % effective in protecting 3–5 day piglets obtained from seronegative sows against challenge. This experiment (De Diego et al. 1994)

established that persistence in the gut and therefore a longer-term mucosal response in older animals can be achieved by using an attenuated live vaccine which replicates in the gut.

A variety of viruses have been used as vectors for expression of full length and truncated versions of S-antigen. Baculovirus-infected cells containing the N-terminal fragment of the S-antigen induced neutralizing serum antibodies in piglets (Tuboly et al. 1994). Furthermore, oral immunization of piglets using S-antigen-recombinant porcine adenovirus induced both neutralizing antibodies in sera and mucosal antibodies in the intestine (Tuboly et al. 2001). Fusions of the S-protein expressed in *E. coli* were immunogenic and produced cognate antibodies but did not neutralize the virus. S-protein expressed in vaccinia virus produced neutralizing antibodies when injected into animals (Hu et al. 1985). The expression of S-antigen in nuclear polyhedrosis virus and its expression in insect s9 cells allowed the production of a secreted version which was immunogenic in rats despite lack of proper glycosylation (Godet et al. 1991). Mucosal and serum antibodies were also elicited in rabbits orally inoculated with an attenuated strain of *Salmonella* Typhimurium expressing a fusion of the D-epitope of the S-antigen to *E. coli* heat-labile toxin B-subunit (Lt-B) (Smerdou et al. 1996).

Various TGEV antigens have been produced in plants. Gomez et al. (1998) expressed either the N-terminal 750 residues, or the entire S-protein, under the control of the CaMV 35S promoter in *Arabidopsis*. The antigen was purified from leaves and administered to mice. The mice produced antibodies that reacted specifically with TGEV and neutralized infective particles. Subsequently, the same group expressed the N-terminal domain in potatoes and obtained a serum response to both intraperitoneally delivered tuber extracts and orally administered tubers. Immunoprecipitation and ELISA results showed that antibodies were detecting the native protein; however, the sera did not neutralize the virus in vitro (Gomez et al. 2000). Tuboly et al. successfully produced neutralizing serum antibodies in piglets using tobacco plants expressing various permutations of the S gene under the control of a synthetic super promoter (Tuboly et al. 2000).

8.3 Technical Progress

8.3.1 Achievement of High Levels of Expression of TGEV-S Antigen

In an early experiment using S-antigen, Gomez et al. (1998) used a CaMV 35S promoter to drive expression of an un-optimized sequence in *Arabidopsis*. While the protein produced was immunogenic, its levels were too low to be visualized by SDS-PAGE and could only be detected by Western blotting. Subsequently, Tuboly et al. (2000) used plant-optimized S-antigen sequences driven by a super promoter and increased levels to 0.1–0.2 % total soluble protein (TSP) S-antigen expression in tobacco.

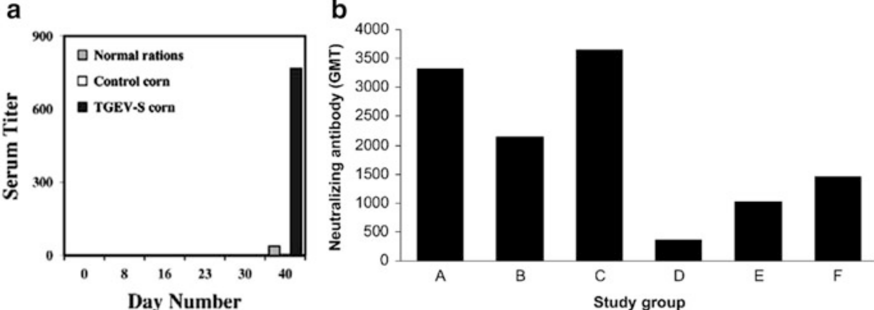

Fig. 8.3 (**a**) Induction of TGEV-neutralizing antibodies in serum from 10–12-day-old piglets fed transgenic corn seed expressing the S-protein of TGEV. Reproduced with permission from Lamphear et al. (2002). (**b**) Colostrum TGEV neutralization titers on the day of farrowing. Pregnant gilts received the following treatments: group A (oral corn vaccine on days −35 to −29 and −14 to −8), group B (oral corn vaccine on days −35 to −33 and −14 to −12), group C (oral corn vaccine on days −35 and −14), group D (oral corn placebo on days −14 to −8), group E (intramuscular live vaccine on days −35 and −14), and group F (oral corn vaccine on days −14 to −8), where the "−" sign represents days before farrowing. Reproduced with permission from Lamphear et al. (2004). See text (Sect. 8.3.2) for discussion

Using codon-optimized sequences for maize fused to the barley alpha-amylase signal sequence (BAASS), Lamphear et al. (2004) achieved levels of S-antigen expression in maize seed of 13 mg/kg. BAASS targets protein to the cell wall and is later cleaved—a process that permits higher levels of accumulation in the apoplast than is possible in the cytoplasm. Using standard plant techniques, F1 lines of maize that had high levels of expression were selected and backcrossed to commercial maize lines to obtain stable, increased levels of protein expression. Since the protein was produced in maize, a GRAS plant, further purification before oral administration was not necessary.

8.3.2 Induction of Neutralizing Antibodies

Lamphear et al. (2002) studied the levels of serum neutralizing antibodies induced by administration of TGEV-S corn alone in piglets. 10–12-day-old TGEV seronegative piglets were divided into three groups (normal rations, control corn, TGEV-S corn) of four piglets each, with 2 mg of the antigen administered in ground corn mixed with medicated milk replacer. 8 days after the last antigen administration, the piglets were challenged with 1 mL of orally administered virulent TGEV. Serum was collected and neutralizing antibody levels measured (Fig. 8.3a). Piglets fed TGEV-S corn showed more than tenfold greater neutralizing serum response as well as milder symptoms (a geometric mean titer of 768.5 in TGEV-S-corn fed piglets compared to titers of 64 in the control groups). While the presence of serum antibodies is significant, greater protection will potentially be afforded by mucosal antibodies.

The induction of neutralizing antibodies in both serum and colostrum was examined in gilts following the administration of oral TGEV vaccine in maize comprising a subunit vaccine of the S-protein expressed in corn (Lamphear et al. 2004). Two kg doses of corn containing 26 mg of antigen were administered to gilts previously sensitized with three doses of modified live vaccine (MLV-TGEV, Intervet) with two oral administrations 115 and 102 days before farrowing and one intramuscular injection 88 days before farrowing. Subsequently, they were divided into six groups for testing (A–F; see Fig. 8.3b) and administered oral TGEV-S corn or control corn at various schedules. TGEV-S corn was given to group A (oral corn vaccine on days −35 to −29 and −14 to −8), group B (oral corn vaccine on days −35 to −33 and −14 to −12), group C (oral corn vaccine on days −35 and −14), group D (oral corn placebo on days −14 to −8), group E (intramuscular live vaccine on days −35 and −14), and group F (oral corn vaccine on days −14 to −8), where the "−" sign represents days before farrowing. Serum antibody levels dropped by up to half at the time of farrowing but were still relatively high compared to control (data not shown). Colostrum obtained on the day of farrowing showed neutralizing antibodies were present. Antibody levels in animals in group C administered test corn for 2 days were almost 3.5 times higher than animals administered intramuscular injection with modified live virus, indicating that corn was more effective at inducing secreted antibody than parenteral vaccine and that the window of administration may be important. That secretory antibodies were produced in colostrum is a clear indication of the stimulation of a mucosal response.

8.3.3 Protection of Piglets Against Challenge with Live Virus

Because it was not clear if the antibodies induced by oral administration were protective against infection, a trial to test protection against challenge was carried out with 10–12-day-old, specific pathogen-free piglets (Streatfield et al. 2001; Lamphear et al. 2002). Piglets were fed TGEV-S corn, control placebo corn, and orally administered MLV-TGEV controls. All piglets were challenged with virulent TGEV on day 18 and monitored twice daily for symptoms for 9 days until the end of the study. Following challenge, piglets were scored twice daily for signs of diarrhea (normal = 0, creamy = 1, watery = 2) and other symptoms (dehydration and depression, anorexia = 1, vomitus = 3, moribund or death = 10) to give a total clinical score. Clinical symptoms for each study group were scored as follows: percent morbidity incidence [(number of animals with clinical signs scoring ≥ 2 divided by total number of animals) $\times$ 100], percent morbidity incidence and duration [(total number of clinical observations $\geq$ divided by the product of the total number of pigs and days scored) $\times$ 100], or clinical severity index (total clinical score divided by the product of the total number of pigs and days scored).

Half of the control group and about 10 % of the MLV-TGEV group showed morbidity, compared to 0 % of the TGEV-S corn (4-day administration) group.

Morbidity duration and clinical severity were highest in control corn, as expected, but TGEV-S corn showed better protection even than MLV-TGEV. Increased duration of administration showed slightly higher morbidity levels (Fig. 8.4), but piglets administered TGEV-S corn for 4 days showed no symptoms.

This unexpected finding suggests the possible induction of oral tolerance to extended exposure of antigen for longer periods than 4 days. The levels of humoral and secreted (serum and stool) antibodies in these piglets were not monitored, but it seems likely that the levels were diminished by the extended exposure. This is an important result, as it indicates that a shorter duration of administration of oral vaccine is more effective and more economical as well.

8.3.4 Tissue Targeting and Stability of Antigen in Maize Seed

S-antigen expression in maize seed was directed by a constitutive polyubiquitin promoter and the protein was present in endosperm, aleurone, and embryonic tissues. Whole seed expressed antigen at a level of 25 μg/g. Fractionation to determine the sites of protein accumulation within the seed showed that the embryo (germ tissue) accumulated the highest concentration of recombinant protein of 50 μg/g—double the concentration of whole grain. The levels in bran (aleurone and pericarp layers) were very low, and endosperm contained levels in between those in grain and pericarp (Lamphear et al. 2002).

Stability of antigen was examined in whole grain stored for 10 months at ambient temperature without humidity monitoring, at 10 °C in a seed storage facility at 50 % humidity, and in corn meal stored at 4 °C (Fig. 8.5). Levels of antigen in stored grain were compared to freshly harvested grain, and no difference was found. This important result indicates that antigen is stable even under uncontrolled conditions at ambient temperature, making it less onerous for storage and transport over long periods and permitting elimination of the cold chain for transport and delivery.

8.3.5 What Expected or Unexpected Hurdles Were Overcome to Reach Target

TGEV-S antigen was not a novel target for expression, but several hurdles that limited its efficacy were overcome. Maize, as the bioencapsulating agent, presumably prolonged exposure to GALT tissues. High levels of expression and stability were achieved using a constitutive promoter and an apoplast targeted BAASS sequence. The antigen was stable in stored grain, overcoming a major economic hurdle.

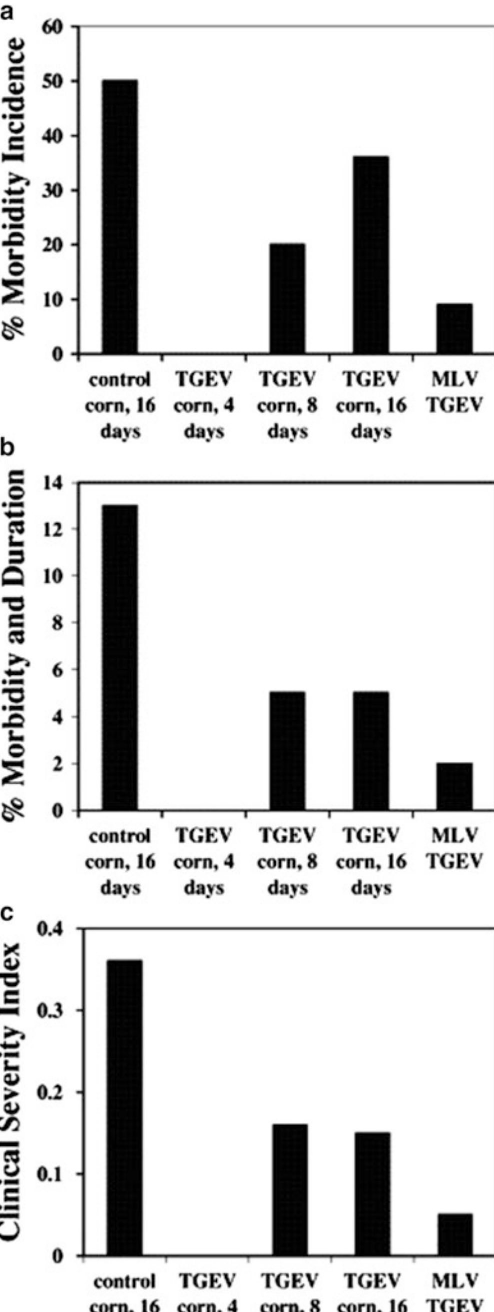

Fig. 8.4 Protection against TGEV of 10–12-day-old specific pathogen-free piglets fed with either control corn or transgenic corn expressing the S-protein (TGEV corn) for 4, 8, or 16 days as indicated. Positive controls were administered modified live vaccine (MLV-TGEV) orally. The panels show: (**a**) percent morbidity incidence, (**b**) percent morbidity incidence and duration, and (**c**) clinical severity index. See text for description of methods. Reproduced with permission from Lamphear et al. (2002)

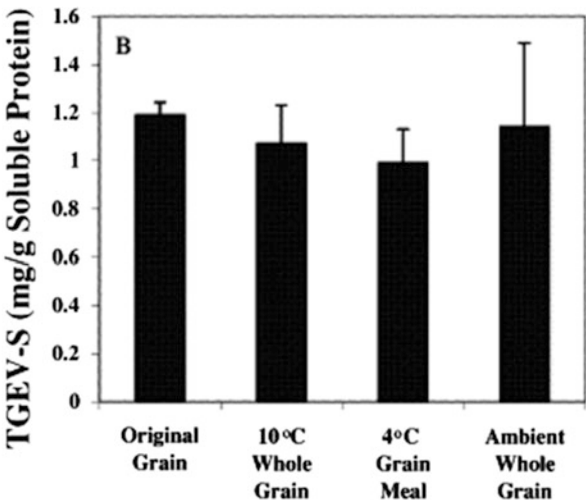

Fig. 8.5 Antigen stability in tissues from transgenic corn seed: measurement of extracted TGEV-S antigen as mg antigen per g extracted soluble protein from grain or grain meal stored for 10 months at either 4 °C, 10 °C, or at ambient temperature in a grain storage facility in Iowa. Values represent mean ± one standard deviation. Figure reproduced with permission from Lamphear et al. (2002)

In this work, it was also unexpectedly discovered that the smallest dose regimens, administered purely by the oral route, were also the most effective. Significantly, when young pigs were fed a constant diet of the antigen for 2 weeks, protection was reduced, an indication that tolerance may develop with long-term exposure (Lamphear et al. 2002). The duration of protection for piglets following antigen administration in feed was not tested. Administration of antigen over a 4-day period gave the highest levels of protection against live challenge—even higher than oral vaccination with a modified live virus (Fig. 8.4; see Sect. 8.3.3).

In gilts, as well, the shortest oral regimen of two boosters showed the highest colostrum levels of serum neutralizing antibodies. Thus, if passive immunity transferred by colostrum is protective in suckling pigs, then this regimen would be more economical as well as less onerous. In gilts, colostrum levels of antibodies persisted for about 3–5 days following piglet birth (Lamphear et al. 2004), which provides passive immunity while the piglets developed their gut flora and become suitable subjects for the administration of vaccines in feed.

8.4 Nontechnical Hurdles

8.4.1 Regulatory Compliance

Regulatory approval must be obtained for each step of the process, including compliance for growing transgenic plants to licensure of the vaccine product. Plant-based vaccines have already been approved for use with livestock and with humans. In 2006, the USDA approved a plant-produced vaccine against Newcastle disease virus for administration to fowl by Dow AgroSciences. In 2012, FDA approved the first pharmaceutical product produced in plants, Elelyso™ (taliglucerase alfa), to Protalix Biotherapeutics and Pfizer. Thus, precedents now exist for both human and animal pharmaceuticals produced in plants. The existence of these plant-based vaccines smooths the path for the development and regulatory approval of future plant-based vaccines. Some essential regulatory procedure for growth of transgenic plants and approval of veterinary vaccines in the USA are described below.

8.4.1.1 APHIS Permits

Similar to the requirement for other vaccines produced in yeast or eggs, the plant crop must not inadvertently be intermixed with commodity food. One important feature of the plant production system is that field-grown plants have the potential to pollinate other food or feed crops. The APHIS arm of the USDA issues permits for the growth of transgenic corn. These include a number of growing restrictions to prevent intermixing of the crop inadvertently with other food and feed crops. To this point, the USDA has developed a highly restrictive set of isolation conditions for growing the crop, over and above the standard conditions for producing vaccines, including a one-mile isolation corridor from other corn. As maize pollen is relatively heavy, it normally only pollinates other corn plants within a few meters (Luna et al. 2001) making this an extreme precaution. Nevertheless, as the growing acreage needed for such a product is low (~70,000 acres, or $\leq$0.1 % of the total acreage of corn in the USA to produce two doses of 26 mg of vaccine at 13 mg antigen/kg corn for each of the estimated 65 million swine and assuming a yield of 150 bushels/acre), it is quite reasonable to find isolated areas to grow the crop. After harvesting, the corn can be processed into corn meal, blended to obtain precise dosing, and formulated to the final product.

8.4.1.2 Obtaining USDA Safety Clearance for Marketing Livestock Vaccine

Production of a vaccine is only the first step in the process towards licensing. The vaccine must be safe and effective in order to obtain USDA approval for use through the Veterinary Biologics program. Guidelines are set out by the USDA under the Virus-Serum-Toxin Act (21 U.S.C. 151–159).

8.4.2 Economic Considerations

8.4.2.1 Cost of Production

The cost of production is always a potential limitation for any product to be commercialized. In this case, since the product is produced in corn which is a feed source for pigs, it does not need to be purified thereby reducing a major expense. Furthermore, the corn itself has value as a feed product, further lowering the effective cost of the vaccine. The actual cost of the vaccine can therefore be calculated by the cost to keep the grain segregated from food and feed crops and loss in yield over commodity crops. Both of these costs are further reduced by having high levels of antigen in maize, thereby limiting the amount of corn needed to be grown. In theory and in practice, the cost should be lower than the traditional injected vaccines. The indirect benefit of not having to physically inject the animals provides another value proposition making this even more desirable.

8.4.2.2 Public Acceptance of Transgenics

The first group that needs to accept this product is the swine producers. This is largely dependent on their perceived need for added protection from the disease. Once this hurdle is overcome, there is always the concern over trying any new product. Acceptance by swine farmers, however, should be relatively straightforward since this product should cost less to administer than traditional vaccines.

Even though farmers may accept this relatively readily, the general public is leery of transgenic crops. On a rational basis, this product would not pose a threat to the general public for several reasons: (1) the product will be quickly digested and will not persist in the animal at the time of slaughter; (2) the protein itself is present in the food chain when animals are infected by the virus, and the use of vaccines limits its presence; (3) the protein itself poses no threat as it has no toxicity or enzymatic activity; (4) as a protein, the degradation products are amino acids that are common to all living organisms; (5) as pork is cooked, any protein would be quickly denatured prior to human consumption; and (6) growing maize expressing S-antigen will involve very small acreage, thus a nationwide concern would not be triggered in the event of an inadvertent exposure.

Unfortunately, logical arguments are not always sufficient; undoubtedly a subset of the general public will fear the product for reasons other than safety arguments. This can be due to a general fear of transgenic crops, vaccines, fear of new products, aversion to technology, or other fears. While all of these are considerable hurdles, they can be overcome if the benefits of this product outweigh the risks. In this case, the benefits can be determined by how much of a threat is perceived by the disease and the cost and efficiency of this approach can eliminate the threat.

8.4.3 Barriers to Commercialization

While the goal of most vaccine production in plants is commercialization, the dearth of products on the market speaks to the many barriers that must be crossed before the goal is achieved: (1) the product must be made at a level in plants that makes it commercially viable; (2) for scale-up with plants such as corn, adequate barriers to dissemination must be incorporated and APHIS approval obtained; (3) the product must be tested in the target animal to rule out negative side effects, and approval for marketing must be obtained; and (4) finally, a large investment to scale-up and market the product is needed. For large companies such as Dow AgroSciences, which take a product from the lab to the market, this is less formidable than for small biotechnology companies which need to move to market at speed lest funding wane while the process is still underway.

8.5 Conclusions

8.5.1 Overall Significance of This Work

Protection with a subunit antigen expressed in corn, exclusively by the oral route, is shown for the first time to be effective in piglets, the target species for immunization. This demonstration, using corn-encapsulated S-antigen administered orally as both primer and booster, could circumvent the need for parenteral vaccinations or oral immunizations with modified live virus, making the process of vaccinating large herds much more economical and less time-consuming than using injected vaccines.

This work has three significant outcomes: (1) the demonstrated use of an oral subunit vaccine in production of neutralizing antibodies in both adults and young pigs, (2) neutralizing antibodies from both active and passive immunity being protective to a direct challenge with live virus, and (3) short duration of oral exposure of antigen (4 days) being sufficient to develop complete protection from challenge.

The use of maize seed that can be administered directly through feed clearly shows that this approach provides protection that can be as good if not better than injectable products. Using antigen-expressing corn as a top dressing on feed has the additional advantage of also bioencapsulating the antigen, which extends its contact with GALT in the gut and provides a greater immune response. Storage stability has also been demonstrated, with both the whole seed and meal, at room temperature and with refrigeration. In short, this approach demonstrates that a practical, low-cost, heat-stable, orally delivered vaccine is achievable.

8.5.2 Additional Improvements That May Make This Product More Valuable

Production of subunit antigens in well-tolerated, edible plant tissue opens the door to a lot of possibilities. Several antigens to different diseases can be combined into a single vaccine by standard breeding techniques. Since antigens expressed in maize seed are tolerant to storage over long periods at ambient temperature, they can be stockpiled against zoonotic outbreaks. The elimination of the cold chain can be highly significant in rural areas. The use of the edible vaccine as both primary and booster increases convenience and lowers duration of administration to animals. Adjuvants can be co-expressed as needed to improve immunogenicity. Clearly, there is ample room for improvement of this technology.

8.6 Future Directions

The use of TGEV-S protein clearly shows commercial potential as described above. Using more recent technology, improvements could be made to produce a higher concentration of the antigen or having it targeted specifically to the embryo. This study also represents one of the first clear demonstrations of providing protection against a pathogen in animals, paving the way for other vaccine antigens to be tested.

References

Bailey MR (2000) A model system for edible vaccination using recombinant avidin produced in corn seed. Texas A&M University, College Station, TX

Belouzard S, Millet JK, Licitra BN, Whittaker GR (2012) Mechanisms of coronavirus cell entry mediated by the viral spike protein. Viruses 4(6):1011–1033

Bosch D, Schots A (2010) Plant glycans: friend or foe in vaccine development? Expert Rev Vaccines 9(8):835–842

Buchholz U, Müller MA, Nitsche A, Sanewski A, Wevering N, Bauer-Balci T, Bonin F, Drosten C, Schweiger B, Wolff T (2013) Contact investigation of a case of human novel coronavirus infection treated in a German hospital, October-November 2012. Euro Surveill 18(8):pii: 20406

Carter JE III, Langridge WHR (2002) Plant-based vaccines for protection against infectious and autoimmune diseases. Crit Rev Plant Sci 21(2):93–109

Correa I, Jiménez G, Suñé C, Bullido MJ, Enjuanes L (1988) Antigenic structure of the E2 glycoprotein from transmissible gastroenteritis coronavirus. Virus Res 10(1):77–93

Daniell H, Streatfield S, Wycoff K (2001) Medical molecular farming: production of antibodies, biopharmaceuticals and edible vaccines in plants. Trends Plant Sci 6(5):219–226

De Diego M, Rodríguez F, Alcaraz C, Gómez N, Alonso C, Escribano JM (1994) Characterization of the IgA and subclass IgG responses to neutralizing epitopes after infection of pregnant sows

with the transmissible gastroenteritis virus or the antigenically related porcine respiratory coronavirus. J Gen Virol 75(10):2585–2593
Delventhal S, Hensel A, Petzoldt K, Pabst R (1992) Effects of microbial stimulation on the number, size and activity of bronchus-associated lymphoid tissue (BALT) structures in the pig. Int J Exp Pathol 73(3):351–357
Desai PN, Shrivastava N, Padh H (2010) Production of heterologous proteins in plants: strategies for optimal expression. Biotechnol Adv 28(4):427–435
Egelkrout E, McGaughey K, Keener T, Ferleman A, Woodard S, Devaiah S, Nikolov Z, Hood E, Howard J (2013) Enhanced expression levels of cellulase enzymes using multiple transcription units. Bioenergy Res 6(2):699–710
Foss DL, Murtaugh MP (2000) Mechanisms of vaccine adjuvanticity at mucosal surfaces. Anim Health Res Rev 1(1):3–24
Garwes D, Lucas M, Higgins D, Pike B, Cartwright S (1979) Antigenicity of structural components from porcine transmissible gastroenteritis virus. Vet Microbiol 3(3):179–190
Ghaderi D, Zhang M, Hurtado-Ziola N, Varki A (2012) Production platforms for biotherapeutic glycoproteins. Occurrence, impact, and challenges of non-human sialylation. Biotechnol Genet Eng Rev 28(1):147–176
Godet M, Rasschaert D, Laude H (1991) Processing and antigenicity of entire and anchor-free spike glycoprotein S of coronavirus TGEV expressed by recombinant baculovirus. Virology 185(2):732–740
Gomez N, Carrillo C, Salinas J, Parra F, Borca M, Escribano J (1998) Expression of immunogenic glycoprotein S polypeptides from transmissible gastroenteritis coronavirus in transgenic plants. Virology 249(2):352–358
Gomez N, Wigdorovitz A, Castanon S, Gil F, Orda R, Borca M, Escribano J (2000) Oral immunogenicity of the plant derived spike protein from swine-transmissible gastroenteritis coronavirus. Arch Virol 145(8):1725–1732
Hofer U (2013) Novel coronavirus spreads. Nat Rev Microbiol 11(4):222. doi:10.1038/nrmicro2998
Hood EE, Devaiah SP, Fake G, Egelkrout E, Teoh K, Requesens DV, Hayden C, Hood KR, Pappu KM, Carroll J, Howard JA (2012) Manipulating corn germplasm to increase recombinant protein accumulation. Plant Biotechnol J 10(1):20–30. doi:10.1111/j.1467-7652.2011.00627.x
Hu S, Bruszewski J, Smalling R, Browne JK (1985) Studies of TGEV spike protein gp195 expressed in E. coli and by a TGE–vaccinia virus recombinant. Adv Exp Med Biol 185:63–82
Jiménez G, Correa I, Melgosa MP, Bullido MJ, Enjuanes L (1986) Critical epitopes in transmissible gastroenteritis virus neutralization. J Virol 60(1):131–139
Jongsma MA, Bolter C (1997) The adaptation of insects to plant protease inhibitors. J Insect Physiol 43(10):885–895
Kong Q, Richter L, Yang YF, Arntzen CJ, Mason HS, Thanavala Y (2001) Oral immunization with hepatitis B surface antigen expressed in transgenic plants. Proc Natl Acad Sci U S A 98(20):11539–11544
Lamphear B, Streatfield S, Jilka J, Brooks C, Barker D, Turner D, Delaney D, Garcia M, Wiggins B, Woodard S (2002) Delivery of subunit vaccines in maize seed. J Control Release 85(1–3):169–180
Lamphear B, Jilka J, Kesl L, Welter M, Howard J, Streatfield S (2004) A corn-based delivery system for animal vaccines: an oral transmissible gastroenteritis virus vaccine boosts lactogenic immunity in swine. Vaccine 22(19):2420–2424
Laude H, Gelfi J, Lavenant L, Charley B (1992) Single amino acid changes in the viral glycoprotein M affect induction of alpha interferon by the coronavirus transmissible gastroenteritis virus. J Virol 66(2):743–749
Luna V, Figueroa M, Baltazar M, Gomez L, Townsend R, Schoper J (2001) Maize pollen longevity and distance isolation requirements for effective pollen control. Crop Sci 41(5):1551–1557
Ma JKC, Drake PMW, Christou P (2003) The production of recombinant pharmaceutical proteins in plants. Nat Rev Genet 4(10):794–805. doi:10.1038/Nrg1177

Mamedov T, Ghosh A, Jones RM, Mett V, Farrance CE, Musiychuk K, Horsey A, Yusibov V (2012) Production of non-glycosylated recombinant proteins in Nicotiana benthamiana plants by co-expressing bacterial PNGase F. Plant Biotechnol J 10(7):773–782

Mari A, Ooievaar-de Heer P, Scala E, Giani M, Pirrotta L, Zuidmeer L, Bethell D, Van Ree R (2008) Evaluation by double-blind placebo-controlled oral challenge of the clinical relevance of IgE antibodies against plant glycans. Allergy 63(7):891–896

Massonneau A, Condamine P, Wisniewski J-P, Zivy M, Rogowsky PM (2005) Maize cystatins respond to developmental cues, cold stress and drought. Biochim Biophys Acta 1729(3):186–199

Masters PS (2006) The molecular biology of coronaviruses. Adv Virus Res 66:193–292

Naqvi S, Ramessar K, Farré G, Sabalza M, Miralpeix B, Twyman RM, Capell T, Zhu C, Christou P (2011) High-value products from transgenic maize. Biotechnol Adv 29(1):40–53

Nuttall I, Dye C (2013) The SARS wake-up call. Science 339(6125):1287–1288

Pabst R (1992) Is BALT a major component of the human lung immune system? Immunol Today 13(4):119–122

Pabst R, Gehrke I (1990) Is the bronchus-associated lymphoid tissue (BALT) an integral structure of the lung in normal mammals, including humans? Am J Respir Cell Mol Biol 3(2):131–135

Reguera J, Ordoño D, Santiago C, Enjuanes L, Casasnovas JM (2011) Antigenic modules in the N-terminal S1 region of the transmissible gastroenteritis virus spike protein. J Gen Virol 92(5):1117–1126

Sestak K, Saif LJ (2008) Porcine coronaviruses. In: Morilla A, Yoon K-J, Zimmerman JJ (eds) Trends in emerging viral infections of swine. Iowa State Press, Ames, IA, pp 321–330. doi:10.1002/9780470376812.ch10a

Sethuraman N, Stadheim TA (2006) Challenges in therapeutic glycoprotein production. Curr Opin Biotechnol 17(4):341–346

Smerdou C, Anton IM, Plana J, Curtiss R, Enjuanes L (1996) A continuous epitope from transmissible gastroenteritis virus S protein fused to *E. coli* heat-labile toxin B subunit expressed by attenuated Salmonella induces serum and secretory immunity. Virus Res 41(1):1–9

Streatfield S, Jilka J, Hood E, Turner D, Bailey M, Mayor J, Woodard S, Beifuss K, Horn M, Delaney D (2001) Plant-based vaccines: unique advantages. Vaccine 19(17–19):2742–2748

Suzuki YA, Kelleher SL, Yalda D, Wu L, Huang J, Huang N, Lönnerdal B (2003) Expression, characterization, and biologic activity of recombinant human lactoferrin in rice. J Pediatr Gastroenterol Nutr 36(2):190–199

Tuboly T, Nagy E, Dennis J, Derbyshire J (1994) Immunogenicity of the S protein of transmissible gastroenteritis virus expressed in baculovirus. Arch Virol 137(1):55–67

Tuboly T, Yu W, Bailey A, Degrandis S, Du S, Erickson L, Nagy E (2000) Immunogenicity of porcine transmissible gastroenteritis virus spike protein expressed in plants. Vaccine 18(19):2023–2028

Tuboly T, Yu W, Bailey A, Erickson L, Nagy E (2001) Development of oral vaccine in plants against transmissible gastroenteritis virus of swine. In: Toutant J, Balazs E (eds) Molecular farming. Proceedings of the OECD workshop held in La Grande Motte, France, 3–6 Sept 2000. Institut National de la Recherche Agronomique, pp 239–248

Verma D, Moghimi B, LoDuca PA, Singh HD, Hoffman BE, Herzog RW, Daniell H (2010) Oral delivery of bioencapsulated coagulation factor IX prevents inhibitor formation and fatal anaphylaxis in hemophilia B mice. Proc Natl Acad Sci U S A 107(15):7101–7106

Weiner HL (1997) Oral tolerance: immune mechanisms and treatment of autoimmune diseases. Immunol Today 18(7):335–343

Weiner HL, da Cunha AP, Quintana F, Wu H (2011) Oral tolerance. Immunol Rev 241(1):241–259

Yamada T, Ohta H, Shinohara A, Iwamatsu A, Shimada H, Tsuchiya T, Masuda T, Takamiya K-i (2000) A cysteine protease from maize isolated in a complex with cystatin. Plant Cell Physiol 41(2):185–191

Chapter 9
Edible Rabies Vaccines

Elizabeth Loza-Rubio and Edith Rojas-Anaya

9.1 Introduction to Rabies Virus

Rabies is derived from the Latin *rabere*, "to rage or to rave," as is the corresponding adjective "rabid"; *rabere* possibly may have earlier origins in Sanskrit *rhabas* for "violence." Since antiquity, rabies has been one of the most feared diseases. Human rabies remains an important public health problem in many developing countries (Wilkinson 2002; Woldehiwet 2002).

The World Health Organization (WHO) reports that more than 60,000 people die of this disease every year (WHO 2013). Most of these cases occur in the developing countries. In most countries of Latin America, the major reservoirs are the dog and, lately, the hematophagous bat (*Desmodus rotundus*), which is present in tropical and subtropical areas from Northern Mexico to Northern Argentina and Chile and transmits the disease mainly to cattle (Loza-Rubio et al. 2005; Delpietro et al. 2009). Vampire bat attacks on cattle are a major concern for cattle-raising areas. Blood loss and paralytic rabies due to bat bites can impose severe losses on the livestock industry (Arellano-Sota 1988).

Any tome which focuses upon some of the major rabies issues spanning the geographical extent from the US/Mexico border to Tierra del Fuego is long overdue, not least because Latin America is rich with historical, cultural, ecological, and viral diversity. One can only speculate about the primordial state of this disease, before canine rabies was imported during the sixteenth century with European colonization.

Clearly, the region has the greatest known diversity of rabies virus variants associated with the *Chiroptera* (the evolutionary well spring of the genus *Lyssavirus*), with representatives of major hosts among at least three bat families.

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Additionally, since the beginning of the twentieth century, a complex epizootiological relationship was identified between rabies viruses and hematophagous bats, leading to bovine paralytic rabies—unique in the entire globe. Similarly, only in the New World are non-human primates (e.g., marmosets in Brazil) believed to serve primary rabies virus reservoirs.

The *Lyssavirus* genus encompasses 15 viruses: rabies virus (RABV), Lagos bat virus (LBV), Mokola virus (MOKV), Duvenhage virus (DUVV), European bat lyssavirus 1 and 2 (EBLV 1 and 2), Australian bat lyssavirus, Aravan virus, Khujand virus, Irkut virus, West Caucasian virus (WCV), and Shimoni bat virus (SHIBV) (Loza-Rubio et al. 2012a, b; Kuzmin et al. 2010; Dietzgen et al. 2011). Another, two new *Lyssavirus* have been identified. One has been isolated from an insectivorous bat (*Myotis nattereri*) in Germany identified as Bokeloh (Freuling et al. 2011), and the other has been isolated from an African civet identified as Ikoma (IKOV) (Marston et al. 2012).

A new tentative *Lyssavirus*, Lleida bat lyssavirus, was found in a bent-winged bat (*Miniopterus schreibersii*) in Spain. It does not belong to phylogroup I or II, and it seems to be more closely related to the WCV bat virus and especially to the Ikoma lyssavirus (Aréchiga Ceballos et al. 2013). Classification of the genus is presented in Table 9.1.

Although several types of *Lyssavirus* are recognized worldwide, currently in the Americas only genotype 1 has been identified, even though there are several groups carrying out epidemiological surveillance in order to verify this situation or if at one point in time any other has been identified (Loza-Rubio et al. 2012a, b).

Rabies virus is the prototype species of the genus *Lyssavirus* in the family *Rhabdoviridae*. This RNA virus contains five genes which codified for the same number of proteins. The five structural proteins of the virion include a nucleocapsid (N), phosphoprotein (P, N, or NS), matrix protein (M), RNA polymerase (L), and a glycoprotein (G) (Fig. 9.1) (Schnell et al., 2010).

9.2 Introduction to Glycoprotein (G)

9.2.1 Structure of G Protein

Rabies virus G protein is a transmembrane protein with 505 amino acids that weighs 65–67 kDa (kda) (Ross et al. 2008) and forms spicules that project outward from the infected cell forming trimers. This protein is used by the virus to join with the host cells and initiates the relationship between them when the cell receptors link. Amino acids 1 through 439 are responsible for the attachment of the virus to the cell receptors causing a fusion of the viral and cell membranes (Gaudin et al. 1993; Gaudin et al. 1999). The three protein-type membrane receptors for the rabies virus that have been identified are (1) the nicotinic receptor for acetylcholine, (2) the low-affinity neurotrophin receptor, and (3) the neural cell

Table 9.1 Classification, geographical distribution, and species affected by *Lyssavirus* genus

Virus	SIGLAS	Maintenance hosts	Geographical distribution
Rabies virus (RV)	VRAB	Carnivora and multiple species of insectivorous and hematophagous bats	Worldwide (except some islands)
Lagos bat virus (LBV)	LBV	Bats	Africa
Mokola virus (MOKV)	MOKV	Humans, cats, dogs, rodents, shrew	Africa
Duvenhage virus DUVV	DUVV	Insectivorous bat	Africa
European bat lyssavirus 1 EBLV-1	EBLV-1	Insectivorous bat (*Eptesicus pipistrellus*)	Europe
European bat lyssavirus 2 EBLV-2	EBLV-2	Insectivorous bat (*Myotis* spp.)	Europe
Australian bat lyssavirus (ABLV)	ABLV	Insectivorous and frugivorous bats (suborder: *Megachiroptera*/*Microchiroptera*)	Australia
Aravan virus (ARAV)	ARAV	Insectivorous bat (*Myotis blythii*)	Asia central
Khujand virus (KHUV)	KHUV	Insectivorous bat (*Myotis mystacinus*)	Asia central
Irkut virus (IRKV)	IRKV	Insectivorous bat (*Murina leucogaster*)	Este de Siberia
West Caucasian bat virus (WCBV)	WCBV	Insectivorous bat (*Miniopterus schreibersii*)	Región del Cáucaso, Asia central
Shimoni	BBLV	Unconfirmed—single isolate from *Hipposideros commersoni* (Commerson's leaf-nosed bat)	Africa
Bokeloh		Insectivorous bat (*Myotis nattereri*)	Europe
Ikoma		Civet	Africa
Lleida		Bent-winged bat *Miniopterus schreibersii*	Spain

Source: Based on Food and Agriculture Organization of the United Nations (2011). Investigating the role of bats in emerging zoonoses: Balancing ecology, conservation and public health interests. Edited by Newman SH, Field HE, de Jong CE, and Epstein JH. FAO

adhesion molecule (NCAM). These receptors are implicated in the adsorption of the rabies virus and the promotion of infection directly into the nerve ends and/or gangliosides located in neurons or at the point of axoplasmic transport of muscles. The virus moves along the dorsal ganglions and spinal cord; the brain is quickly infected causing apoptosis of the nerve cells and T cells (Lafon 2011). A total of five antigenic sites have been identified in the soluble region of the protein: I, II, III, IV, and "a," which are located in residues 231, 330–338, 264, and 342–343, respectively. The antigenic site II, located in position 34–42 and 198–200, had

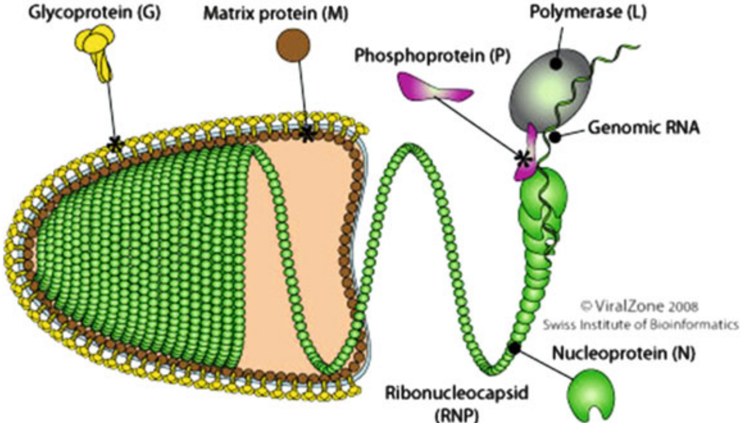

Fig. 9.1 Structure of rabies virus. The five structural proteins of the virion include a nucleocapsid (N), phosphoprotein (P, N or NS), matrix protein (M), RNA polymerase (L), and glycoprotein (G) (Schnell et al. 2010). Source: http://viralzone.expasy.org/viralzone/all_by_species/22.html

only been recognized under denaturing conditions (Benmansour et al. 1991). Furthermore, the presence of a residue of the Glu amino acid in position 333 has been related with the invasion of neurons and pathogenicity (Wunner 2002); the lack of this amino acid causes nonrecognition of the virus and the strains become nonpathogenic.

It has been proposed that amino acid changes within the antigenic sites of the G protein could be the source of variants that are capable of escaping the host's defenses and providing adaptation to new environments (Kobayashi et al. 2010; Khawplod et al. 2006).

9.2.2 *Immunogenic Activity of G Protein*

The G protein is a target of T lymphocytes and induces the formation of neutralizing antibodies against the virus (Loza-Rubio et al. 1998; Morales et al. 2006). This is the reason why this protein has been used for making vaccines, since it is the most exposed antigen of the virus.

The immune response that is triggered by the rabies virus is peculiar due to the immuno-privileged condition of the nervous system. This is based mainly on the restriction of T-cell migration and the deficiency in professional antigen-presenting cells (Lafon 2005). Protection against the rabies virus is mediated by the production of virus-neutralizing antibodies and/or T lymphocytes (helper CD4+ and cytotoxic CD8+). Although it is possible that the protection against an infection by the rabies virus is the result of various effector-host interactions, virus-neutralizing antibodies (VNA), which are mostly produced against the G protein, play an important role in

the immunological protection against a rabies infection (Dietzschold et al. 1990; Desmeziéres et al. 1999). Furthermore, the G protein, besides inducing the formation of virus-neutralizing antibodies, also promotes the production of helper and cytotoxic T cells. It has been shown in intracerebral challenges that its structure is critical for both actions, the induction of neutralizing antibodies and protection (Drings et al. 1999; Hooper et al. 1994). The function of these T cells during rabies infection is to help in the induction of B cells and the production of antibodies, as well as to act as cell effectors in cytotoxic cell immunity response (Jackson 2003). After capture by macrophages and other antigen-presenting cells, antigens of the rabies virus are presented to CD4 or CD8 cells. This stimulation induces the production of cytokines such as IL-2, IL-4, and IFN-γ (Drings et al. 1999).

Regarding the immune response developed after vaccination, it involves the activation of specific differentiated B cells within plasma that produce antibodies and memory B cells. Produced antibodies are specific against the G protein (antibodies against other proteins are also generated with live or inactive virus vaccines) and are directed specifically against the antigenic components of the G protein and neutralize the virus (Lafon et al. 1990). Production of these virus-neutralizing antibodies involves a refined process of specificity adjustment which results in the selection of antigen avid cells. This specificity adjustment and antibody production depend on the correct folding of the protein; otherwise, the antigenic sites do not become exposed (Desmeziéres et al. 1999).

9.2.2.1 Heterologous G Protein Expression for Use in Immunizations

Because G protein induces antibodies against the rabies virus, this protein can be used as an immunogen when expressed in vectors such as Vaccinia, Canarypox, adenovirus, and yeast and in DNA vaccines, as well as in transgenic plants (Cadoz et al. 1992; Kieny et al. 1984; Xiang et al. 1996; Henderson et al. 2009; Sakamoto et al. 1999; Tacket 2009; Ventini et al. 2010).

The need for a safer and more effective vaccine has promoted the development of oral vaccines. In the case of wild rabies, specifically in foxes, the first massive oral vaccination was carried out in 1978 within the Rhône Valley in Switzerland and later it was extended to various other territories. This distribution was carried out manually using vaccine-laden bait (12–25 bait/km^2) within endemic zones (Wandeler 2000, Bugnon et al. 2004). The first recombinant vaccine was the VR-G developed in 1984 in which the sequence of the G gene was inserted into a plasmid together with the Vaccinia promoter and flanked by the viral thymidine kinase gene. This plasmid was used to transfect cells that had been previously infected with a wild Vaccinia strain, and using homologous recombination, it was possible to obtain the recombinant plasmid (Kieny et al. 1984; Paolazzi et al. 1999. Oral immunization of foxes has allowed the large-scale elimination of the virus in areas of Europe where the baits have been placed. Oral vaccination with the vaccines Raboral V-RG (Vaccinia recombinant virus expressing G protein) (Kieny et al. 1984) and with Rabigen SAG2 (double mutant avirulent strain

SAG2) (Artois et al. 1992) was effective for wild rabies in Europe which has helped to almost eradicate wild rabies in the western part of the continent (Desmettre et al. 1990) and has been successful for rabies control in Canada, the United States, and other countries (Lontai 1997; Mainguy et al. 2013). Nevertheless, until now, there is no effective oral vaccination for reservoir species. Notably, in the United States, there are no licensed vaccines for skunks.

Adenoviruses have been used as vectors for the expression of the G protein, promoting neutralizing antibodies and providing protection against intracerebral challenges in lactating mice that came from vaccinated mothers but that had no tolerance. It has been reported that this construct has higher efficacy when compared to conventional and VR-G vaccines (Xiang et al. 1996; Wang et al. 1997; Tims et al. 2000; Hu et al. 2006). In fact, its efficacy has been studied in dogs; in this species the efficacy can be compromised if there are antibodies against the same adenovirus, but only if the inoculation is intramuscular (Yuan et al. 2008). This prototype showed in cats the same efficacy to a challenge carried out at 12 months. Furthermore, a vaccine prototype using a type 2 adenovirus that expressed the rabies virus glycoprotein has been recently evaluated in sheep in which the said construct showed promising results when inoculated through either intramuscular or intradermal pathways (Bouet-Cararo et al. 2010).

Poxviruses have also been used as vectors for the expression of several antigens that induce both the cellular and the humoral responses. Avian poxvirus (Canarypox) has been preferred, since it does not infect humans, for the expression of the rabies G protein. It is known as the ALVAC-RG and has been shown to generate antibodies and promote protection immunity in cats and dogs. This vaccine is well tolerated in humans producing results that are at least similar to those of the diploid cell vaccine (Fries et al. 1996).

Some attenuated strains of Aujeszky's disease virus have been used as vector for the G gene of the rabies virus. This prototype has shown its efficacy in dogs when administered through various pathways, including intramuscular, but most importantly the oral pathway (Yuan et al. 2008).

Furthermore, the baculovirus, which infects insects, has also been used since it allows a high expression of proteins. Tordo and colleagues reported in 1993 the cloning of the Mokola 3 genotype which was used as a vaccine (Tordo et al. 1993). This construct protected mice against a lethal challenge. In addition, the efficacy of a baculovirus that contained the G and N genes of the rabies virus was evaluated and its effectiveness was demonstrated. In another study the ectodomain of the rabies G gene was cloned into this system and compared with a DNA vaccine. The recombinant baculovirus induced antibody titers that were higher than the other vaccine.

The first demonstration that a plasmid could carry a protection antigen (G gene) of the rabies virus was published in 1994 (Xiang et al. 1996). This group showed that this immunogen was capable of promoting a protecting immune response when challenged in primates that were immunized with this vaccine and were capable of surviving a rabies virus challenge, while in others the DNA vaccine caused long-term protection levels through the production of neutralizing antibodies after a

single immunization (Lodmell and Ewalt 2000). This group has also reported that 10 mg of G protein-codifying plasmid inoculated via intramuscular injection protects 100 % of mice, while the intradermal injection of 0.1 mg protects up to 83 %.

It also has been shown that DNA vaccines work in species such as dogs, cats, and horses (Osorio et al. 1999; Perrin et al. 2000; Fischer et al. 2003). Also, using normal syringes, various inoculation pathways have been evaluated such as intramuscular and intradermal (Perrin et al. 2000; Lodmell et al. 2006; Osinubi et al. 2009).

9.3 Description of the Systems Used to Produce the Protein

9.3.1 Theoretical Advantages of the Plant Process over Other Technologies

Literature indicates several potential advantages that are related to plant-derived vaccines, for example, heat-stable formulation for storage and transport (avoiding cold chain) which is important in tropical and subtropical areas and ease of delivery for better compliance leading to a reduced demand for skilled health-care professionals in developing and developed countries.

The use of recombinant gene technologies by the vaccine industry has revolutionized the way antigens are generated and has provided safer, more effective means of protecting host organisms against bacterial, viral, and parasitic pathogens (Lamphear et al. 2002; Loza-Rubio and Gomez-Lim, et al 2006) (Table 9.2).

In the case of viruses, no alternative to vaccines exists for animals since there are no antiviral drugs suitable for widespread application in the field. This underlines the need for controlling viral diseases of animals by vaccination. Advances in genetic engineering have made it possible to insert heterologous genes into several plant species, such as cereals and legumes. Plants are increasingly recognized as legitimate systems for the production of recombinant proteins and antigens. A wide range of proteins have been expressed and used for diagnostic purposes, industrial and pharmaceutical production of enzymes, food additives, therapeutic proteins, antibodies, and vaccine antigens (Streatfield 2006). However, despite nearly 20 years of development, there are only two plant-produced vaccine-related products that have gone all the way through all production and regulatory hurdles (Rybicki 2009).

Table 9.2 Advantages and disadvantages of the various types of vaccines including recently developed ones

Vaccine type	Advantages	Disadvantages	Cost of production
Live, attenuated	– Strong immune response	– Parenteral administration (syringes) – Prolonged immunity with one or two doses – Can give rise to the virulent form of the pathogen – Must be refrigerated in order to maintain their potency – Possible spread of the pathogen into the environment	300–10,000 USD
Inactivated or dead	– Safer and more stable than live ones – Immune response compare to live ones	– Immune response weaker than live vaccines – Require regular boosters – Parenteral administration (syringes)	300–10,000 USD
Viral vectors – Simulate a natural infection, strong immune response – Can generate a response against more than one pathogen – Sophisticated infrastructure	– Can be administrated via mucosa – Their development requires much time due to the genetic construction of the vector – It has not been fully established if the DNA is not integrated into the host's genome	– Complicated purification processes	*Not reported
Recombinant DNA	– Can be administrated via mucosa – Produce strong cell and antibody response – Relatively easy to produce – Do not necessarily need a cold chain for preservation	– Can contain impurities that could be toxic to the host – Use of gene gun – Require boosters since they do not contain the antigen as such – It has not been fully established if the DNA is not integrated into the host's genome – Require purification	>100 USD
Subunits: Bacteria Baculovirus Yeasts	– These are target specific – Safe to produce and handle – Low probability of adverse reactions	– Their development can take a long time – Low immunogenic properties – Require adjuvants – Parenteral administration (syringes)	>100 USD

Source: Boehm (2007), Bower et al. (2009), Peeters et al. (2001), Tillman et al. (2004), Streatfield et al. (2003)

9.3.2 Immunogenicity of Rabies Virus Antigen Expressed in Plants

The G protein, which is the main antigen of the rabies virus, has also been expressed in tomato, tobacco, and spinach plants. The first experience with the expression of the G protein of the rabies virus was in tomatoes (McGarvey et al. 1995). The full G gene of the virus was cloned into the BIN19 vector downstream of the 35S CaMV promoter. Later, tomato cells were transformed by infection with *Agrobacterium tumefaciens*. The expressed glycoprotein was purified by immunoprecipitation from leaves and fruits, and two bands, one of 60 kDa and another of 62 kDa, were detected with Western blot. This variation in protein weight could be due to differential glycosylation in the plant cell. The amount of recombinant glycoprotein in leaves was between approximately 1 and 10 ng/mg of soluble protein, while fruits had lower amounts.

Furthermore, other studies have reported that the oral administration of the rabies virus ribonucleoprotein induces the production of neutralizing antibodies when afterwards an inactive virus vaccine booster is applied in mice (Dietzschold et al. 1987). Also, as a way to improve the expression of proteins in plants, other viruses have been used as vectors to infect plant tissue such as the alfalfa mosaic virus (AIMV) using the coat protein (Cp) which serves a carrier for the peptides to be expressed. In this manner, Yusibov et al. (1997) expressed using this system the G and N proteins of the rabies virus and the human immunodeficiency virus type 1 (HIV-1). These constructs were inoculated into tobacco plants (*Nicotiana benthamiana*) in order to later isolate the virus from the leaves and semi-purify the viral particles for their inoculation of mice. Animals received seven doses (10 μg per dose) via intraperitoneal injection and assessing the response in the presence or absence of adjuvant. Using antibodies against the Cp protein, the presence of a 28.9 kDa band was found in Western blot which corresponds to the fusion protein formed by the Cp protein of AIMV and the viral peptides of the rabies virus. The identification of each peptide was carried out using monoclonal antibodies against each of them. Finally, it was demonstrated that the viral particles that were inoculated promoted an immune response in mice against the rabies virus antigens, as well as those of HIV-1, regardless of the adjuvant was present or not.

In another study, using the constructs reported by Yusibov's group, infection of tobacco and spinach plants was carried out (Modelska et al. 1998). In this study, mice were immunized with protein purified from transformed leaves by oral and intraperitoneal route. Inoculation was carried out using 50 μg of purified recombinant virus in three doses. These same particles were administered orally through gastric intubation in four doses (250 μg per dose). Another group was fed for 7 days with the transformed spinach leaves (1 g per dose containing 15 μg of antigen). In all groups, serum samples and fecal pellets were collected 2 days before each immunization and the neutralizing activity of rabies virus-specific serum antibodies was determined. In animals immunized via intraperitoneal route, the presence of antibodies was observed after the second immunization. The mice immunized by oral route showed the

presence of IgG and IgA. The higher levels of immune response generated by the leaf-feeding approach as compared with gastric intubation raise the possibility that the plant cells enhanced the delivery of virus particles to the sites of immune responses. A total of 40 % of the animals survived the challenge.

Using this same transient expression system in these two previous studies, Yusibov and collaborators in 2002 assessed these plants not only in mice but also in people (Yusivov et al. 2002). In the oral immunity study, three lots of spinach (3,000 plants) were inoculated with the recombinant virus that expresses the peptides of the rabies virus. Mice were immunized via intraperitoneal route with the purified recombinant protein (250 mg = 35 μg of peptide per dose) together with Freund's adjuvant and later challenged. Two groups were formed in the experiment with people. The first group (five individuals) were previously immunized against rabies and then were fed using 20 g of fresh transformed spinach containing 0.6 mg of recombinant virus (84 μg of protein). The second group was composed of nine volunteers without previous immunization who received 150 g of fresh spinach tissue per dose (700 μg of protein).

Afterwards they received a dose of commercial vaccine intramuscularly and the presence of IgG and IgA was determined in serum. The leaves of spinach were found to contain 0.4 ±0.007 mg of recombinant virus in fresh tissue that contained 84 mg of the chimeric peptide. A 19.3 kDa band, corresponding to the fusion peptide, was detected using Western blot. The whole (100 %) of the mice immunized with the extract survived the challenge, 43 % of those immunized with the synthetic peptides, and 20 % of those immunized with the alfalfa mosaic virus. Furthermore, three of the five volunteers mounted an effective response against the antigen after ingesting the transformed spinach. In six of the nine volunteers, the antibody titers increased against the recombinant virus. Four of these individuals showed IgG and 2/7 showed IgA. In 5/9 of the volunteers there was an increase in IgG in serum after receiving three doses of the spinach leaves. In none of these experiments was tolerance observed.

Using tobacco also, Ashraf et al. (2005) expressed the glycoprotein fused to an endoplasmic reticulum retention sequence (SEKDEL) in order to improve its expression. The gene was cloned downstream of a double CaMV35S promoter. Transformation was measured using *Agrobacterium tumefaciens*. The protein was purified and 25 μg of this extract was used to immunize five mice via intraperitoneal receiving boosters at days 7, 14, and 28. These were later challenged using a standard laboratory strain (CVS). The protein purified from plant leaves showed a single band of ~66 kDa corresponding at G protein. Transformed plants contained chimeric G protein at 0.38 % of the total soluble protein. The rabies glycoprotein expressed in tobacco is glycosylated and is not degraded during the purification. These proteins show immunoreactivity to antirabies virus antibodies and elicit a high level of immune response in mice. The plant-derived GP gave 100 % protection similar to the commercial vaccine. In comparison with other studies, the protein that accumulates in tobacco is of higher molecular mass, comparable to the native protein.

In Mexico, edible vaccines have been developed using corn and carrots, proving their efficiency in mice. In some cases these provided 100 % protection in animals when challenged with a lethal virus originating from vampire bats (Lerma 2005; Rojas et al. 2009; Loza-Rubio et al. 2008).

In the first report carried out by our study group, we reported the expression of the gene that codes for the glycoprotein in corn. Corn is a cereal rich in protein which is used for both human and animal consumption; this plant has been an adequate experimental model because of the high levels of expression of transgenes obtained. It was perceived as a species with great potential for producing an edible vaccine. The vector used for the transformation of the plant was pGHCNS. G gene rabies virus was cloned downstream of the promoter and the maize ubiquitin promoter 35ScaMV. The expression cassette was flanked by matrix attachment region (MARs). Maize embryogenic calluses were transformed with the above construction by biolistics. Regenerated maize plants were recovered and grown in greenhouse. The presence of the G gene and its products was detected in vegetal tissue by PCR and Western blot. A fine powder was prepared from transformed grains and administered as pellet (50 μg of recombinant G protein). Other groups of mice were immunized intramuscularly with 50 μg of G protein using a commercial vaccine. All mice were challenged intracerebrally at day 90 post-vaccination using a vampire bat rabies virus which is used to evaluate commercial vaccine in Mexico. Embryogenic calluses were transformed by biolistics and herbicide-resistant plants were obtained. Twenty-five plants were recovered and 92 % contained the G gene as detected by PCR. The rabies G protein was identified by Western blot and presented a size of about 69 kDa. This increase in molecular weight may be due to posttranslational modifications, and this modification does not seem to have any adverse effect on the antigenic properties of the protein. Similar modifications were showed by McGarvey et al. (1995). Protein was expressed at 1 % of total soluble protein, which is equivalent to about 50 μg per gram of fresh weight in mass. This study obtained a higher level of expression than ever reported. The level of expression obtained in this study is comparable to results obtained by others in maize when expressing the spike protein of the transmissible gastroenteritis coronavirus and the fusion protein of Newcastle disease virus (Lamphear et al. 2002; Guerrero-Andrade et al. 2006). In sera, mice were seronegative at the start of the experiment, but by day 90 post-vaccination, titers varied by more than 0.5 IU. The animals were protected at 100 %, similar at the commercial vaccine. This work has demonstrated that the systems for transformation, selection, and regeneration of mice developed in this study are efficient. Likewise, the plant-based G protein was able to induce viral neutralizing antibodies and protect mice after challenge.

In another assay, the glycoprotein was expressed using carrots to be used in the immunization of mice (Rojas et al. 2009). This plant model was used since carrot is a vegetable that is widely distributed and easy to produce and can be consumed raw.

The G gene of the rabies virus arctic fox strain was subcloned between the double enhancer cauliflower mosaic virus 35S promoter and 35S CaMV terminator in the vector pUCpSS; this construct was named pUCpSSrabG. For transformation, we decided to use the minimal cassette expression approach (promoter-gene-terminator).

We employed carrot seeds for induction of carrot callus. We were able to regenerate 300 adult plants from 100 calli selected in liquid medium, and 93.3 % of the analyzed plants showed integration of the transgene with levels of expression varying from 0.2 to 1.4 % TSP. In our project, the plant-produced band migrated slightly above the native G protein (~70 vs 65 kDa); one likely explanation for this was glycosylation of the protein by the plant. For selection, we employed the gene coding for phosphinothricin acetyltransferase (*bar*), which confers resistance to herbicide Basta. Embryogenic calluses were transformed by biolistics and herbicide-resistant plants were obtained in liquid medium. The presence of the G gene in leaves was determined by PCR and the protein was detected by Western blot using rabbit polyclonal serum against rabies G protein. In order to evaluate the carrot as vaccine, 24 mice were divided into four groups: G1, fed standard mouse chow (negative control); G2, received an intramuscular dose of inactivated rabies vaccine; G3, mice fed 50 μg of rabies virus G protein in 2 g of raw carrot; and G4, mice received 50 μg rabies virus G protein contained in 2 g of raw carrot plus 50 μg of N protein rabies virus (N protein was orally administered) since this molecule has been reported as adjuvant in some rabies vaccines. Mice vaccinated were challenged intracerebrally 60 days post-vaccination. We showed that the ingestion of antigen expressed in carrot resulted in protective rabies antibodies (66 %). These results are consistent with previous studies where the glycoprotein of rabies virus was expressed either in tobacco or in spinach. In this study, we did not observe a 100 % protection of the mice; this is possibly because a greater concentration of G protein is necessary. To improve the protective dose, 100 μg (4 g of carrots) could be administered instead of the 50 μg used in this study.

Recently transgenic corn was used supplied under controlled conditions at various dosages in sheep via oral administration of a single dose. The results showed that 2 g of the G protein of the rabies virus protected more than 80 % of the animals challenged with a lethal vampire bat origin virus (Loza-Rubio et al. 2012a, b). This assay used the same conditions for obtaining transformed corn reported previously by Loza-Rubio et al. (2008). Similarly the Basta herbicide was used for selecting transformed plants.

When the plants reached adulthood, kernels expressing the glycoprotein were identified by PCR and Western blot and were subsequently pooled and quantified before immunization. The animals were divided into six groups containing six animals per group as follows: Group 1, sheep fed 0.5 mg of rabies virus G protein in 20 g of ground maize kernels; Group 2, sheep fed 1.0 mg of rabies virus G protein in 40 g of ground maize kernels; Group 3, sheep fed 1.5 mg of rabies virus G protein in 60 g of ground maize kernels; Group 4, sheep fed 2.0 mg of rabies virus G protein in 80 g of ground maize kernels; Group 5, sheep vaccinated with an inactivated rabies vaccine administered intramuscularly; and Group 6, animals fed 40 g of non-transformed ground maize kernels. Once all groups had been immunized, they were deprived of feed and water for 4 h. The animals were bled to evaluate immune response in serum. Sheep were challenged by the injection at 120 days post-vaccination. The G protein was detected slightly above the native G protein (~70 kDa). The same increase in molecular weight was observed in polyacrylamide gel electrophoresis; the differentially expressed band seems to be

heavier than the native G protein (positive control). The expression level obtained from this analysis was an average of 25 μg of G recombinant protein/g of fresh tissue in the three different lines.

At day 30 post-vaccination, rabies virus antibodies were detected in all vaccinated groups. Animals that received one or two doses of antigen (0.5 and 1.0 mg, respectively) showed a survival rate of 50 % (three deaths in six vaccinated animals). In Group 3, which was immunized with 1.5 mg of protein G, only two sheep died of rabies (2/6), with a survival rate of 66 %. The lowest mortality was found among sheep immunized with the commercial vaccine and those receiving 2.0 mg of protein, which protected 83 % of the animals (1/6). In this study, a large amount of protein was needed to elicit an immune response because a significant portion of the recombinant protein was likely degraded in the rumen. Although we did not observe any signs of tolerance, this could be because the sheep were fed the edible vaccine only once. These results demonstrated the effectiveness of the oral immunization of sheep with corn that expresses the rabies virus G protein. This is the first study in which an orally administered edible vaccine has shown efficacy in a polygastric species.

9.3.3 Benchmarks of What Is Needed to Commercialize the Product in This System

This new technology could contribute to global vaccination programs and have a dramatic impact on public and veterinary health, not only in our country but also in others with similar problems. Nevertheless, the fact that transformed corn must not be grown on open fields must be taken into account since it is an open pollination plant. It should be grown in greenhouses with the highest biosecurity. There are also issues that still need to be resolved, such as the antigen dose that each plant produces since it could produce tolerance (Loza-Rubio and Rojas-Anaya 2010). One possible alternative for the production of antigenic proteins using plants as an expression system is the use of suspension plant cell in pellets that express the antigens of interest. This would avoid the need for growing the plants in greenhouses. The administration would be oral, which is a significant advantage that could be of interest to pharmaceutical companies.

9.4 Technical Progress

9.4.1 Improvements to the Production System for a Rabies Virus Vaccine in Plants

The plant systems evaluated by our group were carrots and corn with the best results, in terms of protein expression and antibody production, obtained with corn.

Genetic modification of cereals has been carried out by direct DNA transfer (i.e., the introduction, integration, and expression of foreign genes) into protoplasts or intact cells grown in vitro using polyethylene glycol treatment and electroporation. These methods have their disadvantages, such as low transformation efficiency of monocotyledon plants and expression levels, which make them impractical. Thus, biolistics was a good option for improving the expression of recombinant proteins (Klein and Fitzpatrick-McElligott 1993; Christou 1995). Other factors for obtaining high levels of expression are described below.

This technique allows the introduction of naked DNA (biologically active) into intact plant cells by the acceleration of DNA-covered microparticles (tungsten or gold) through an explosion mechanism (pressure gun) or by gas bursts (carbon dioxide, nitrogen, or helium) (Klein et al. 1988). Biolistics revolutionized the genetic engineering of monocotyledon species, such as corn.

9.4.2 Challenges for the Optimization of Protein Expression

An important aspect for obtaining an edible vaccine is to develop efficient expression levels in terms of total soluble protein ($\geq$1 %) since low percentages (0.01 %) require purification of the protein. There are several strategies for increasing the expression levels such as the optimization of the gene, use of strong promoters (tissue specific), non-translated leading sequences in the 3′ region, subcellular target signals, crossing of transgenic strains with high expression, germplasm crossing, plastid transformation, and the use of vegetable virus expression systems (Mett et al. 2008; Streatfield 2006; Potenza et al. 2004; Walmsley and Arntzen 2000; Sala et al. 2003; Gleba et al. 2005).

9.4.2.1 Use of Specific Promoters

Currently there is a wide range of promoters that have been used for regulating the expression of the transgene in transformed plants. Regulation can occur at any step of the expression process, and the promoters that manage it ensure the said control during transcription. The election of a promoter for plant transformation depends on the objective and purpose for which the plant is being transformed, as well as its species. Promoters that are specific to the species or tissue can be used, as well as those for seeds or grains, flowers, pollen, roots, leaves, or even aerial tissue (Buchanan et al. 2000; Potenza et al. 2004). Within the promoter are regulating regions, sequence motifs, or *cis* elements; these regulating sequences are known as increasers. These can be located upstream or downstream of the coding region. These regions are required to carry out the maximum transcription of a gene, and it is due to this reason that they are used within plant transformation vectors (Alberts et al. 2002).

The most common of the constitutive promoters used in plant transformation is the 35S promoter from the cauliflower mosaic virus (35S CaMV) (Odell et al. 1985), which is highly valued since it has high expression levels at practically all regions of a transgenic plant. This promoter can achieve high expression levels of the transgene both in monocotyledons and in dicotyledonous plants, although in the former it is somewhat recalcitrant (Battraw and Hall 1990; Benfey et al. 1990).

Regarding the expression of the G protein of the rabies virus in corn, the use of matrix attachment regions (MARs) that flanked the expression cassette became relevant, as they allowed the expression of the proteins to become constitutive. In carrots it was decided that a double 35S promoter of the cauliflower mosaic virus within vector pUCpSS was to be used as promoter, while in corn it was decided to use the corn ubiquitin promoter (Ubi). The use of this promoter has demonstrated that it increases the expression levels of heterologous genes in several cereal species (Cornejo et al. 1993; Gallo-Meagher and Irving 1993; Taylor et al. 1993; Vasil 1994). In contrast with other studies which have purified the recombinant proteins, our study did not require such process and remarkably the expression of the G protein ranged from 0.01 % up to 1.4 % of the total soluble protein.

Finally, although the edible vaccine system is attractive and novel, in practice its application can become complicated due to the following reasons:

1. Dosing of the antigen will depend on its expression level within the plant tissue, even though good expression levels have been demonstrated generally. In order to determine this parameter, each plant line produced would need to be analyzed (individual plants).
2. The expression levels that are obtained are not always inheritable to the next generations of the transgenic line. Furthermore, the seeds that develop from these lines can have genotypic and phenotypic characteristics that are undesirable, such as loss of fertility and others.

9.5 Nontechnical Hurdles

9.5.1 Production

One of the most important publications of the Mexican regulations in this issue is the "special protection regime for corn." In the context of plant-derived vaccines, it is important to mention that the regime establishes: "The experimentation or release into the environment of genetically modified corn that has characteristics that prevent or limit its use or consumption by humans or animals shall not be allowed, as well as their use in the processing of food for human consumption." The aforementioned blocks the experimentation with corn in Mexico for the development of antigens and other biopharmaceuticals. This is because Mexico is the origin center and diversity of this cereal. An alternative might be the development of these edible vaccines in plants different to the maize. In other regions, such as the United States and Europe, the development of transgenic corn

is not allowed because the regulation in these countries is more flexible. The regulatory framework of other countries has been discussed by Loza-Rubio and coworkers previously (Loza-Rubio and Rojas-Anaya 2010).

9.5.2 Regulatory

Once the regulatory item be resolved, there are other important points that must be covered regarding the design and elaboration of a vaccine derived from plants, such as assuring transgene stability and antigen expression in the following generations, guaranteeing the consistency and reproducibility of the methods used to obtain the vaccine, evaluation and monitoring of the environment to avoid contamination of endemic species, making sure that efficient methods are available for quantification of the antigen to determine correct dosing and bioavailability of the vaccine, and assessment of the handling and transporting techniques with regard to wastes produced from the transformed plants. Because of all this, legislation is necessary, both at global and local levels, on the research and development of biological products using transgenic plants as a platform. In this regard, Hungary became the first Central European country to adopt legislation on the regulation of genetic engineering activity. Its Gene Technology Law entered into force in January 1999 with the concomitant establishment of an advisory body (The Gene Technology Committee). Subsequently, several countries of Central and Eastern Europe adopted regulations about genetically modified organisms (GMOs).

Before 2000, the differences between the US and the European community in their approach to the regulation of biotechnology had arisen because of the different initial cognitive frameworks, a different level of trust in the government, and the dissimilar agro-political situation. The dissimilar cognitive frameworks arise primarily because many Europeans appear to view the environment as a fragile ecosystem that may be easily unbalanced by transgenic plants. In contrast, the dominant view in the United States is of a resilient environment that can easily adapt. In September 2003, the Cartagena Protocol, an international treaty governing the movements of living modified organisms (LMOs) resulting from modern biotechnology from one country to another, was adopted. One of the principal objectives of the protocol is to provide information to importing countries to assist their decision making when accepting imports of LMOs. Now, boasting almost 190 member governments all around the world (known as “Parties”), the Convention has three goals: the conservation of biodiversity, the sustainable use of the components of biodiversity, and the fair and equitable sharing of the benefits arising from the use of genetic resources (Loza-Rubio and Rojas-Anaya 2010).

During the FAO Global Biotechnology Forum in 2005 (Ruane and Sonino, 2008), countries discussed about the kinds of GMO regulatory systems that might be appropriate for developing countries; it is important to consider that GMOs for food and agriculture are a very heterogeneous group, for example, the potential environmental risks from GM forest trees and the release of a GM yeast to make

bread are different. In addition, within each of these sectors, GMOs may vary considerably, requiring different kinds of regulations, for example:

- Some species are not grown for food (e.g., cotton), so food safety regulations are not strictly an issue although it should be kept in mind that some material, e.g., pollen/honey derived from GM, may still enter the food chain.
- The same species may be modified for very different traits, e.g., an agricultural crop or animal may be modified to produce human pharmaceuticals as tomatoes producing vaccines against virus or animals producing hormones. "Pharmed" products under development include vaccines, antibodies, and industrial proteins and, in the crop sector, involve banana, maize, potato, and tomato plants. Special regulations covering potential gene flow to their conventional counterparts may be necessary;
- Regulations may vary depending on whether the GM species is produced for export or domestic use. For this reason, GMO commercialization is subject to a strict marketability requirement. Otherwise, GMO varieties are not approved for commercialization. When exports are not a significant factor (e.g., in the case of cotton), commercial release can be approved irrespective of the regulatory status elsewhere, since there are no "sensitive" markets for the product.

On the other hand, there are twelve countries that are noteworthy due to their high levels of biodiversity: Brazil, Indonesia, Colombia, Australia, Mexico, Madagascar, Peru, China, the Philippines, India, Ecuador, and Venezuela, known as mega-diverse; therefore, it is very important to preserve their germplasm (Tovar 2008).

The identity of Mexico in terms of its biodiversity is important therefore its global role must be recognized. Mexico is a country with domestic and wild plant species of which it is their center of origin, so their protection has been requested. In this context, Mexico is one of the countries signatory to the Cartagena Protocol that was adopted on 29 January 2000 (Cartagena Protocol, 2000). The majority of the problems related to gene flow corresponding to GMOs and the issues regarding the responsibility/compensation were examined in the legislation framework of the Cartagena Protocol on Biosafety to the Convention on Biological Diversity. Article 1 mentions: "In accordance with the precautionary approach contained in Principle 15 of the Rio Declaration on Environment and Development, the objective of this Protocol is to contribute to ensuring an adequate level of protection in the field of the safe transfer, handling and use of living modified organisms resulting from modern biotechnology that may have adverse effects on the conservation and sustainable use of biological diversity, taking also into account risks to human health, and specifically focusing on transboundary movements."

Before 2005, the Ecological Equilibrium and Environmental Protection General Act (Tovar 2008) was the national judicial instrument that provided the basis for regulation regarding GMOs. It has the objective of "regulating the activities of confined use, experimental release, pilot program release, commercial release, marketing, import and export of genetically modified organisms" in order to prevent, avoid, or reduce the possible risks that these activities could cause to human health, as well as to the health of animals, plants, and water animals, the

environment, and the biological diversity of the country. It establishes as competent authorities for issuing permits and sanctions the Ministry of the Environment and Natural Resources; the Ministry of Agriculture, Livestock, Rural Development, Fisheries and Food; and the Ministry of Health. It also establishes the basis for the operation of the Inter-ministry Commission on Biosafety for Genetically Modified Organisms (CIBIOGEM) through which the various aforementioned ministries must collaborate regarding the biosafety of GMO.

The law established that a permit will be required for carrying out the following activities (Ley de bioseguridad de Organismos Geneticamente Modificados, 2005):

(a) Experimental release into the environment, including imports for this activity, of one or more GMOs
(b) Release into the environment in a pilot program, including imports for this activity
(c) The commercial release into the environment, including imports for this activity, of GMOs

In order to establish a risk assessment of the aforementioned points, each case is to be analyzed individually through scientific and technical studies carried out by the interested parties, evaluating the possible risks of the experimental release into the environment and to the biological diversity, as well as to the health of animals, plants, and fisheries. The studies must include possible risks to human health. Up to the development of this document, no reference has been made regarding transformed plants that express an antigen (plant-derived antigens).

The latest amendment to the Regulations of the Genetically Modified Organisms Biosafety Act was published after 2009. These regulations establish, among other things:

(a) The characteristics that must be contained within the request for permission to carry out activities using GMOs
(b) The requirements for permits for release into the environment
(c) Considerations on the import and export of GMOs that are destined for their release into the environment
(d) Characteristics of the Internal Biosafety Commissions of public and private institutions
(e) Determination of the centers of origin and genetic diversity
(f) Establishment of the National Biosafety Information System
(g) Determination of the list of GMOs that are to be issued by the competent ministries

On the other hand, Brazil, another mega-diverse country with great advances and biotechnology development, promulgated Decree 6.041 of the Policy on the Development of Biotechnology in which the objective is “To promote and carry out actions in order to establish the adequate environment for developing biotechnology products and innovative processes, promote the greatest efficiency of the national productive structure, the innovative capacity of Brazilian companies, the adsorption of technologies, the generation of business and the expansion of exports (Ley No. 1.105, 2005; Biotechnology Development Policy 2007).”

In comparison with the Mexican regulation, it also established the competences of the ministries regarding activities with GMOs, the integration of Biosafety Committees, etc. It is noteworthy that in the said document, it is established as a strategic objective to stimulate the production of recombinant proteins using plants, animals, and microorganisms as bioreactors and the plants resistant to biotic and abiotic stress. The aforementioned emphasizing on the coexistence of transgenic and conventional varieties promoting the development of mechanisms and technologies for preserving the genetic identity of cultivars, as well as the development of geographical information systems for monitoring and zoning of the activities related to distance biotechnology safety.

Recent developments in genetic modification and the use of LMOs in agriculture have ignited a debate over the potential effects of these organisms on biological diversity. The regime does allow states to enact national protective measures to preserve human and animal health as well as natural resources, based on scientific evidence. However, it is necessary to ensure that this is not only on paper but is carried out in order to avoid ecological imbalances that could affect all species including humans.

9.5.3 Public Perception

One of the main challenges that modern biotechnology currently has is the acceptance by consumers of the products developed by it, especially of products derived from transgenic plants for their use as food. This is known as "biotechnophobia," which is the rejection of anything that has been derived from biotechnology due to its denomination as something "not natural" and/or "potentially dangerous." Unfortunately, this has been promoted by ecologist associations that have a strong penetration in mass media. It is important that scientific institutions and community promote the use of everyday language to describe the benefit of new technological discoveries. For example, the use of the word "transgenic" is perceived as something that is against nature, and in some cases, it has been used instead of "transgender" in mass media causing further confusion. Another such word that should be avoided is "mutation" since it is associated with an organism that causes harm and that it is against nature, association that comes about due to its use in science fiction.

9.6 Conclusions

The use of biolistics as a method for plant genetic transformation and the use of plant expression vectors with constitutive promoters helped to achieve the development of carrot and corn plants that express the G protein of the rabies virus. These plants were successful antigen production systems. According to the results obtained in this study, it was found that the G protein expressed in the plant

systems under evaluation was functional, even though it had suffered some posttranslational modifications. When immunity assays were carried out, it was found that corn tissue was the most effective in providing greater protection when challenged with rabies virus from hematophagous bats, which are the main transmitter of this virus in Mexico. Results show that both carrot and corn are convenient systems for the expression of the G protein since good expression levels were achieved and such levels would allow the production of a subunit vaccine. Nevertheless, the expression of the N protein in tomato plants was not satisfactory. It is noteworthy that the use of biolistics allows permanent expression since the transgene is integrated into the plant genome and from there it segregates into future generations thus obtaining a good candidate for an edible vaccine against the rabies virus in animals and humans.

This study allows us to visualize a rabies vaccine derived from plants since it was demonstrated that plant cells from both species under evaluation are capable of expressing the G protein of the virus in sufficient quantities. It is recommended that further studies be carried out on wild animals, which are transmitters of the disease.

Commercial production of vaccine of this type depends on overcoming regulatory frameworks on the use of plant cells as protection antigen producers without the need for using a whole plant as the biological medium.

References

Alberts B, Jonson A, Lewis J, Raff M, Roberts K, Walter P (2002) Molecular biology of the cell, 4th edn. Garland Science, New York

Aréchiga Ceballos N, Vázquez Morón S, Berciano JM, Nicolás O, Aznar López C, Juste J, Rodríguez Nevado C, Aguilar Setién A, Echevarría JE (2013) Novel lyssavirus in bat, Spain. Emerg Infect Dis 19:793–795

Arellano-Sota C (1988) Vampire-bat transmitted rabies in cattle. Rev Infect Dis 10:707–709

Artois M, Guittré C, Thomas I, Leblois H, Brochier B, Barrat J (1992) Potential pathogenicity for rodents of vaccines intended for oral vaccination against rabies: a comparison. Vaccine 10:524–528

Ashraf S, Singh PK, Yadav DK, Shahnawaz M, Mishra S, Sawant SV, Tuli R (2005) High level expression of surface glycoprotein of rabies virus in tobacco leaves and its immunoprotective activity in mice. J Biotechnol 119:1–14

Battraw MJ, Hall TC (1990) Histochemical analysis of CaMV 35S promoter-B- glucuronidase gene expression in transgenic rice plants. Plant Mol Biol 15:527–538

Benfey PN, Ren L, Chua N-H (1990) Tissue-specific expression from CaMV 35S enhancer subdomains in early stages of plant development. EMBO J 9:1677–1684

Benmansour A, Leblois H, Coulon P, Tuffereau C, Gaudin Y, Flamand A, Lafay F (1991) Antigenicity of rabies virus glycoprotein. J Virol 65:4198–4203

Biotechnology Development Policy (2007) National Biotechnology Committee. Brazil

Boehm R (2007) Bioproduction of therapeutic proteins in the 21st century and the role of plants and plant cell as production platforms. Ann NY Acad Sci 102:121–131

Bouet-Cararo C, Contreras V, Fournier A, Jallet C, Guilbert JM, Dubois E, Thiery R, Bréard E, Tordo N, Richardson J, Scwartz-Cornil I, Ziéntara S, Klonjkowoki B (2010) Canine adenoviruses elicit both humoral and cell-mediated immune responses against rabies following immunization of sheep. Vaccine 29:1304–1310

Bower DM, Kristala L, Prather J (2009) Engineering of bacterial strains and vectors for the production of plasmid DNA. Appl Microbiol Biotechnol 82:805–813

Buchanan BB, Gruissem W, Jones RL (eds) (2000) Biochemistry and molecular biology of plants. American Society of Plant Physiologists, Rockville, MD, pp 340–342

Bugnon P, Breitenmoser U, Peterhans E, Zanoni R (2004) Efficacy of oral vaccination in the final stage of fox rabies elimination in Switzerland. J Vet Med 51:433–437

Cadoz M, Strady A, Meigner B, Taylor J, Tartaglia J, Paoletti E et al (1992) Immunisations with canarypox virus expressing rabies glycoprotein. Lancet 339:1429–1432

Cartagena protocol on biosafety to the convention on biological diversity. United Nations. Montreal, 2000

Christou P (1995) Particle bombardment. In: Galbraith DW, Bourque DP, Bohnert HJ (eds) Methods in cell biology, vol 50. Academic, St. Louis, MO, pp 375–382

Cornejo M, Luth D, Blankenship KM, Anderson OD, Blenchl AE (1993) Activity of a maize ubiquitin promoter in transgenic rice. Plant Mol Biol 23:558–567

Delpietro HA, Lord RD, Russo RG, Gury-Dhomen F (2009) Observations of sylvatic rabies in Northern Argentina during outbreaks of paralytic cattle rabies transmitted by vampire bats (*Desmodus rotundus*). J Wildl Dis 45:1169–1173

Desmettre P, Languet B, Chappuis G, Brochier B, Thomas I, Lecocq L-P, Kieny M- P, Blancou J, Aubert M, Artois M, Pastoret P-P (1990) Use of vaccinia recombinant for oral vaccination of wildlife. Vet Microbiol 23:227–236

Desmeziéres E, Jacob Y, Saron MF, Delpeyroux F, Tordo N, Perrin P (1999) Lyssavirus glycoproteins expressing immunologically potent foreign B cell and cytotoxic T lymphocyte epitopes as prototypes for multivalent vaccines. J Gen Virol 80:2343–2351

Dietzgen RG, Calisher CH, Kurath G, Kusmin IV, Rodríguez LL, Stone DM (2011) Family Rhabdoviridae. In: King AM, Adams MJ, Carstens EB, Lefkowitz EJ (eds) Virus taxonomy: classification and nomenclature of viruses. Ninth Report of the International Committee on Taxonomy of Viruses. Elsevier, San Diego

Dietzschold B, Wang H, Rupprecht C, Celis E, Tollis M, Ertl H, Heber-Katz E, Koprowski H (1987) Induction of protective immunity against rabies by immunization with rabies virus ribonucleoprotein. Proc Natl Acad Sci U S A 84:9165–9169

Dietzschold B, Gore M, Casali P, Ueki Y, Rupprecht CE, Notkins AL, Koprowski H (1990) Biological characterization of human monoclonal antibodies to rabies virus. J Virol 64:3087–3090

Dioario Oficial de la Federación (2009) Reglamento de la Ley de Bioseguridad de Organismos Geneticamente Modificados

Drings A, Jallet C, Chambert B, Tordo N, Perrin P (1999) Is there an advantage to including the nucleoprotein in a rabies glycoprotein subunit vaccine? Vaccine 17:1549–1557

Fischer L, Minke J, Dufay N, Baudu P, Audonnet JC (2003) Rabies DNA vaccine in the horse: strategies to improve serological responses. Vaccine 21:4593–4596

Food and Agriculture Organisation of the United Nations (2011) Investigating the role of bats in emerging zoonoses: balancing ecology, conservation and public health interests. In: Newman SH, Field HE, de Jong CE, Epstein JH (eds). FAO Animal Production and Health

Freuling CM, Beer M, Conraths FJ, Finke S, Hoffmann KB, Kiemt J, Mettenleiter TC, Mühlbach E, Teifke J, Wohlsein P, Müller T (2011) Novel lyssavirus in Nattere's bat Germany. Emerg Infect Dis 17:1519–1522

Fries LF, Tartaglia J, Taylor J, Kauffman EK, Meignier B, Paoletti E, Plotkin S (1996) Human safety and immunogenicity of canarypox-rabies glycoprotein recombinant vaccine and alternative poxvirus vector system. Vaccine 14:428–434

Gallo-Meagher M, Irving JE (1993) Effects to tissues type and promoter strength on transient GUS expression in sugar cane following particle bombardment. Plant Cell Rep 12:666–670

Gaudin Y, Ruigrok RW, Knossow M, Flamand A (1993) Low-pH conformational changes of rabies virus glycoprotein and their role in membrane fusion. J Virol 67:1365–1372

Gaudin Y, Moreira S, Benejean J, Blondel D, Flamand A, Tuffereau C (1999) Soluble ectodomain of rabies virus glycoprotein expressed in monomeric conformation that is antigenically distinct from the native state of the complete, membrane-anchored glycoprotein. J Gen Virol 80:1647–1656

Gleba Y, Klimyuk S, Marillonet S (2005) Magnifection: a new platform for expressing recombinant vaccines in plants. Vaccine 23:2042–2048

Guerrero-Andrade O, Loza-Rubio E, Olivera-Flores MT, Feérvari-Bone T, Gómez-Lim MA (2006) Expression of the Newcastle disease virus fusion protein in transgenic maize and immunological studies. Transgenic Res 15:455–463

Henderson H, Jackson F, Bean K, Panasuk B, Niezgoda M, Slate D et al (2009) Oral immunization of raccoons and skunks with a canine adenovirus recombinant rabies vaccine. Vaccine 27:194–197

Hooper DC, Pierrard L, Modelska A, Otvos L, Fu Z, Koprowski H, Dietzschold B (1994) Rabies nucleocapsid as an oral immunogen immunological enhancer. Proc Natl Acad Sci U S A 91:10908–10911

Hu R, Zhang S, Fooks AR, Yuan H, Liu Y, Li H, Tu C, Xia X, Xiao Y (2006) Prevention of rabies virus infection in dogs by a recombinant canine adenovirus type-2 encoding the rabies virus glycoprotein. Microbes Infect 8:1090–1097

Jackson AC (2003) Rabies virus infection: an update. J Neurovirol 9:253–258

Johnson N, Cunningham AF, Fooks AR (2010) The immune response to rabies virus infection and vaccination. Vaccine 28:3896–3901

Khawplod P, Shoji Y, Ubol S, Mitmoonpitak C, Wilde H, Nishizono A, Kurane I, Morimoto K (2006) Genetic analysis of dog rabies viruses circulating in Bangkok. Infect Genet Evol 6:235–240

Kieny MP, Lathe R, Drillien R, Spenhner D, Skory S, Schmitt D et al (1984) Expression of rabies virus glycoprotein from a recombinant vaccinia virus. Nature 312:163–166

Klein TM, Fitzpatrick-McElligott S (1993) Particle bombardment: a universal approach for gene transfer to cells and tissues. Curr Opin Biotechnol 4:583–590

Klein TM, Fromm M, Weissinger A, Dwight T, Schaaf S, Sletten M, Sanford JC (1988) Transfer of foreign genes into intact maize cells with high-velocity microprojectiles. Proc Natl Acad Sci U S A 85:4305–4309

Kobayashi Y, Suzuki Y, Itou T, Carvalho AA, Cunha EM, Ito FH, Gojobori T, Sakai T (2010) Low genetic diversities of rabies virus population within different host in Brazil. Infect Genet Evol 10:278–283

Kuzmin IV, Mayer AE, Niezgoda M, Markotter W, Agwanda B, Breiman RF, Rupprecht CE (2010) Shimoni bat virus, a new representative of the Lyssavirus genus. Virus Res 149:197–210

Lafon M (2005) Modulation of the immune response in the nervous system by rabies virus. CTMI 289:239–258

Lafon M (2011) Evasive strategies in rabies virus infection. Adv Virus Res 79:33–53

Lafon M, Edelman I, Bouvet JP, Lafange M, Martchatre E (1990) Human monoclonal antibodies specific for the rabies virus glycoprotein and N protein. J Gen Virol 71:1689–1696

Lamphear BJ, Streatfield SJ, Jilka JM, Brooks CA, Barker DK, Turner DD, Delaney DE, Garcia M, Wiggins B, Woodard SL, Hood EE, Tizard IR, Lawhorn B, Howard JA (2002) Delivery of subunit vaccines in maize seed. J Control Release 85:169–180

Lerma BSL (2005) Caracterización en plantas de maíz obtenidas a partir de callos embriogénicos transformados con el gen de la proteína G del virus de la rabia. Master Thesis. CINVESTAV-Irapuato. Marzo

Ley de Bioseguridad de Organismos Geneticamente Modificados (2005) Diario Oficial de la Federación. México

Ley No. 1.105 decreto No. 5.591 Normas de seguridad y mecanismos de fiscalización de actividades relacionadas con OGM. Brasil. 2005

Lodmell DL, Ewalt CL (2000) Rabies vaccination: comparison of neutralizing antibody responses after priming and boosting with different combinations of DNA, inactivated o recombinant vaccinia virus vaccines. Vaccine 18:2394–2398

Lodmell DL, Ewalt LC, Parnell MJ, Rupprecht CE, Hanlon C (2006) One-time intradermal DNA vaccination in ear pinnae one year prior to infection protects dogs against rabies virus. Vaccine 24:412–416

Lontai I (1997) The current state of rabies prevention in Europe. Vaccine 15S:S16–S19

Loza-Rubio E, Gomez-Lim MA (2006) De Pasteur a nuestros días, batalla contra la rabia. Ciencia y Desarrollo 176:26–33

Loza-Rubio E, Rojas-Anaya E (2010) Vaccine production in plant systems–an aid to the control of viral diseases in domestic animals: a review. Acta Vet Hung 58:511–522

Loza-Rubio E, Pedroza-Requénes R, Montano-Hirose JA, Aguilar Setién A (1998) Caracterización con anticuerpos monoclonales de virus de la rabia aislados de fauna doméstica y silvestre de México. Vet Mex 29:345–349

Loza-Rubio E, Rojas AE, Banda RVM, Nadin-Davis SA, Cortez GB (2005) Detection of multiple strains of rabies virus RNA using primers designed to target Mexican vampire bat variants. Epidemiol Infect 133:927–934

Loza-Rubio E, Rojas AE, Gómez NL, Olivera FMTJ, Gómez-Lim M (2008) Development of an edible rabies vaccine in Maize using the Vnukovo strain. Dev Biol (Karger) 131:477–482

Loza-Rubio E, Nadin-Davis SA, Morales SE (2012a) Molecular and biological properties of rabies viruses circulating in Mexican Skunks: focus on P gene variation. Rev MexCiencPecu 3:155–170

Loza-Rubio E, Rojas AE, Gómez NL, Flores OMT, Gómez-Lim MA (2012b) Protein G from rabies virus expressed in maize protects mice against a challenge with an heterologous rabies virus. Vaccine 30:5551–5556

Mainguy J, Fehlner-Gardiner C, Slate D, Rudd RJ (2013) Oral rabies vaccination in raccoons: comparison of ONRAB and RABORAL V-RG vaccine-bait field performance in Québec, Canada and Vermont, USA. J Wildl Dis 49:190–193

Marston DA, Horton DL, Ngeleja C, Hampson K, McElhinney LM, Banyard AC, Haydon D, Cleaveland S, Rupprecht CE, Bigambo M, Fooks AR, Lembo T (2012) Ikoma lyssavirus, highly divergent novel Lyssavirus in an African Civet. Emerg Infect Dis 18:664–667

McGarvey PB, Hammond J, Dienelt MM, Hooper CD, Fu FZ, Dietzschold B et al (1995) Expression of the rabies virus glycoprotein in transgenic tomatoes. Biotechnology 13:1484–1487

Mett V, Farrance EC, Green BJ, Yusibov V (2008) Plants as biofactories. Biologicals 36:354–358

Modelska A, Dietzschold B, Fleysh N, Fu ZF, Steplewski K, Hooper C et al (1998) Immunization against rabies with plant-derived antigen. Proc Natl Acad Sci USA 95:2481–2485

Morales MM, Rico RG, Gómez OJ, Aguilar SA (2006) Importancia inmunológica de la proteína N en la infección por virus de la rabia. Rev Vet Méx 37:352–364

Odell J, Nagy F, Chua N-H (1985) Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. Nature 313:810–812

Osinubi MOV, Wu X, Franka R, Niezgoda M, Nok AJ, Ogunkoya AB, Rupprecht CE (2009) Enhancing comparative rabies DNA vaccine effectiveness through glycoprotein gene modifications. Vaccine 27:7214–7218

Osorio JE, Tomlinson RS, Frank EJ, Haanes K, Rushlow JR, Haynes JR, Stinchcomb DT (1999) Immunization of dogs and cats with a DNA vaccine against rabies virus. Vaccine 17:1109–1116

Paolazzi CC, Pérez O, De Filipo J (1999) Rabies vaccine. Mol Biol 11:137–147

Peeters K, Wilde CHD, Jeager G, Angenon G, Depicker A (2001) Production of antibodies and antibody fragments in plants. Vaccine 19:2756–2761

Perrin P, Jacob Y, Aguilar SA, Loza-Rubio E, Jallet C, Aubert M et al (2000) Immunization with DNA vaccine induces protection against rabies virus. Vaccine 18:479–486

Potenza C, Aleman L, Sengupta-Gopalan C (2004) Targeting transgene expression in research, agricultural, and environmental applications: promoters used in plant transformation. In Vitro Cell Dev Biol-Plant 40:1–22

Rojas AE, Loza-Rubio E, Flores OMT, Gómez-Lim MA (2009) Expression of rabies virus G protein in carrots (*Daucus carota*). Transgenic Res 18:911–919

Ross BA, Favi MC, Vásquez V (2008) Glicoproteína del virus rábico: Estructura, inmunogenicidad y rol en la patogenia. Rev Cil Infect 25:S14–S18

Ruane J, Sonino A (2008) Results from the FAO biotechnology forum. FAO, Rome

Rybicki EP (2009) Third International conference on plant-based vaccines and antibodies. Expert Rev Vaccines 8:1151–1155

Sakamoto S, Ide T, Tokiyoshi S, Nakao J, Hamada F, Yamamoto M et al (1999) Studies on the structures and antigenic properties of rabies virus glycoprotein analogues produced in yeast cells. Vaccine 17:205–218

Sala F, Rigano M, Barbante A, Basso B, Walsley AM, Castiglione S (2003) Vaccine antigen production in transgenic plants: strategies, gene constructs and Perspectives. Vaccine 21:803–808

Schnell MJ, McGettigan JP, Wirblich C, Papaneri A (2010) The cell biology of rabies virus: using stealth to reach the brain. Nat Rev Microbiol 8:51–61

Streatfield SJ, Lane JR, Brooks CA, Barker DK, Poage ML, Mayor JM, Lamphear BJ et al (2003) Corn as production systems for human and animal vaccines. Vaccine 21:812–815

Streatfield SJ (2006) Engineered chloroplasts as vaccine factories to combat bioterrorism. Trends Biotechnol 24:339–342

Tacket C (2009) Plant-based oral vaccines: results of human trials. Curr Top Microbiol Immunol 332:103–117

Taylor MG, Vasil I, Vasil IK (1993) Enhanced GUS expression in cereal/grass cell suspension and immature embryos using the maize ubiquitin-based plasmid pAHC25. Plant Cell Rep 12:491–495

Tillman U (2004) Advances in the production of human therapeutic proteins in yeast and filamentous fungi. Nat Biotechnol 22:1409–1414

Tims T, Briggs DJ, Davis RD, Moore SM, Xiang Z, Ertl HCJ, Fu ZF (2000) Adult dogs receiving a rabies booster dose with a recombinant adenovirus expressing rabies virus glycoprotein develop high titer of neutralizing antibodies. Vaccine 18:2804–2807

Tordo N, Bourhy H, Sather S, Ollo R (1993) Structure and expression in baculovirus of the Mokola virus glycoprotein: an efficient recombinant vaccine. Virology 194:59–69

Tovar MP (2008) Ley General del Equilibrio ecologico y la Protección al Ambiente. In: Bioseguridad en la amplicación de la Biotecnología y el uso de los organismos geneticamente modificados. CIBIOGEM. pp 287

Vasil IK (1994) Molecular improvement of cereals. Plant Mol Biol 25:925–937

Ventini DC, Astray RM, Jemos MA, Jorge SA, Riquelme CC, Suazo CA et al (2010) Recombinant rabies virus glycoprotein synthesis in bioreactor by transfected Drosophila melanogaster S2 cells carrying a constitutive or an inducible promoter. J Biotechnol 146:169–172

Walmsley AM, Arntzen CJ (2000) Plants for delivery of edible vaccines. Curr Opin Biotechnol 11:126–129

Wandeler AI (2000) Oral immunization against rabies: afterthoughts and foresight. Scheweiz Ach Tierheilk 142:455–462

Wang Y, Xiang Z, Pasquini S, Erlt HC (1997) The use an E1-deleted, replication defective adenovirus recombinant expressing the rabies virus glicoproteína for early vaccination of mice against rabies virus. J Vilol 71:3677–3683

Wilkinson L (2002) History. In: Jackson CA, Wunner HW (eds) Rabies. Elsevier, San Diego, CA, pp 1–21

Woldehiwet Z (2002) Rabies: recent developments. Res Vet Sci 73:17–25

World Health Organization (2013) WHO expert consultation on rabies. No. 982. Geneva

Wunner WH (2002) Rabies virus. In: Jackson AC, Wunner WH (eds) Rabies, 2nd edn. Academic, San Diego, pp 23–68
Xiang ZQ, Yang Y, Wilson JM, Ertl HCJ (1996) A replication-defective human adenovirus recombinant serves as highly efficacious vaccine carrier. Virology 219:220–227
Yuan Z, Zhang S, Liu Y, Zhang F, Fooks RA, Li Q, Rongliang H (2008) A recombinant pseudorabies virus expressing rabies virus glycoprotein: safety and immunogenicity in dogs. Vaccine 26:1314–1321
Yusibov V, Modelska A, Steplewski K, Agadjanyan M, Weiner D, Hooper C, Koprowski H (1997) Antigens produced in plants by infection with chimeric plant viruses immunize against rabies virus and HIV-1. Proc Natl Acad Sci U S A 94:5784–5788
Yusivov V, Hooper DC, Spitsin SV, Fleysh N, Kean RB, Mikheeva T, Deka D, Karasev A, Cox S, Randall J, Koprowsky H (2002) Expression in plants and immunogenicity of plant virus-based experimental rabies vaccine. Vaccine 20:3155–3164

Chapter 10
Newcastle Disease Vaccines

Miguel A. Gómez Lim

10.1 Introduction

Newcastle disease virus (from now on NDV will be used as a reference to the virus and ND to the disease) is a lethal disease infecting a wide range of both domestic and wild birds in most countries and has a disastrous effect on poultry production (Alexander 1997; Seal et al. 2000). The disease took its name from Newcastle upon Tyne, UK, where it was first described in 1926. Outbreaks of the disease are devastating, with mortality close to 100 % in susceptible birds. It spreads rapidly during epizootics, causing severe economic losses and, for countries that export poultry or poultry products, losses resulting from trade restraints and embargoes. Most of the countries with poultry production have relied on vaccination to keep NDV controlled, but since the disease is enzootic in many parts of the world and in spite of the fact that the virus has been recognized for nearly 90 years and despite all the research and attention, it still represents a major limiting factor and a constant threat for increasing production in many countries, while other countries experience sporadic outbreaks. In this review, the current status of the disease along with the different vaccination approaches will be reviewed. For the reader interested in a more in-depth analysis of the disease, the immune responses to the disease, and the history behind the development of the different vaccines, the review by Alexander et al. (2012) is recommended. The possibility of developing a commercial plant-produced NDV vaccine will be discussed.

10.1.1 The Disease

Although it has been convincingly demonstrated that ND is a new virus disease of poultry (Doyle 1927), some authors originally suggested that the disease was just

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another form of "fowl plague" (pathogenic avian influenza) and early research mainly focused on the demonstration of this hypothesis. For that reason, the disease has been referred to through the years as pseudo-fowl pest, Ranikhet disease, atypical Geflugelpest, pseudo-poultry plague, Korean fowl plague, avian pest, avian distemper, pseudovogel pest, Tetelo disease, and avian pneumoencephalitis (Alexander et al. 2012). The disease is endemic in many parts of the world including countries in Asia, the Middle East, Africa, and Central and South America. Most of the countries in the European Union experience sporadic outbreaks, and the United States and Canada have seen high mortality in wild cormorants caused by the virus. Although international monitoring of ND is carried out by the Food and Agriculture Organization of the United Nations (FAO) and the World Organization for Animal Health (OIE), figures may not represent the true distribution of the disease.

Because of this worldwide impact, most of those working in the field would have considered ND as the single most significant disease of poultry (Alexander et al. 2012). However, in the past 10–15 years, it has been overshadowed by the emergence and spread of highly pathogenic avian influenza (HPAI) virus of subtype H5N1 across Asia and Europe and into Africa. Although the HPAI H5N1 virus has become endemic in some countries and still causes spontaneous outbreaks in others, the acute alarm caused throughout the veterinary and medical fields has subsided to some extent, and attention is once more being given to the other devastating disease of poultry, ND.

Morbidity and mortality rates can vary greatly depending on the virulence of the virus strain and susceptibility of the host. Environmental conditions, secondary infections, vaccination history, and avian species all affect these rates. The disease affects both domestic and wild bird populations. In chickens, morbidity can be up to 100 % with 90 % mortality. In other species, such as finches and canaries, clinical signs may not be present. A carrier state may exist in psittacine and some other wild birds. Ducks and geese may be infected and show few or no clinical signs, even with strains lethal for chickens. In many countries, poultry is raised in large farms using intensive management systems which crowds together thousands of birds in a closed, warm, and dusty environment. These living conditions may be highly conducive to the transmission of the disease (Meszaros 1983), apart from causing undue stress to the animals (Hughes et al. 1989) and complications by malnutrition (Ben-Nathan et al. 1981), factors that increase susceptibility to the disease (Mohamed and Hanson 1980).

10.1.2 The Newcastle Disease Virus

The causative agent of the disease is the Newcastle virus, also called paramyxovirus aviar type 1, which is a member of the *Paramyxoviridae* family, of the *Avulavirus* genus, subfamily *Paramyxovirinae*. There are nine avian paramyxovirus serotypes designated APMV-1 to APMV-9. Of these, Newcastle disease virus, which is APMV-1, remains the most important pathogen for poultry, but APMV-2, APMV-3,

APMV-6, and APMV-7 may also cause disease in poultry. Strains are classified into three pathotypes based on their virulence in chickens.

To date, a large variety of strains of the NDV have been identified and, regardless of the origin, all are positive for hemagglutinin inhibition using polyclonal antiserum against NDV, prepared against a strain of reference of the same virus (King 2001). These viruses possess an RNA genome of a single chain in negative sense. The size of the genome is 15,186 nucleotides coding for six proteins: the nucleoprotein (NP), the phosphoprotein (P), the matrix protein (M), the fusion protein (F), the hemagglutinin neuraminidase (HN), and an RNA polymerase dependent on RNA (L) (Seal et al. 2000). Furthermore, the proteins V and W are produced by splicing of the gene P. Three proteins are associated with the envelope of the virus, HN and F, which are found inserted into the viral membrane as spikes that extend from 8 to 12 nm on the surface and the M protein which is non-glycosylated and is peripherally associated with the inner surface of the membrane. The other three proteins, NP, P and L, are associated with the genomic RNA forming the nucleocapsid (Morrison 2003). Inside the viral membrane, the nucleocapsid that contains the genome is found, which is associated to the NP forming a nucleus to which the proteins P and L bind (Morrison 2003).

Infection by NDV is initiated in the respiratory or gastrointestinal tract of the chickens. Binding and fusion of the virus to the host cell are directed by two viral glycoproteins, the HN and the F. HN is responsible for binding to the cell receptors that contain sialic acid and has hemagglutinin and neuraminidase activity. The F protein is synthesized as an inactive precursor (F0) that is activated by cell proteinases, resulting in a polypeptide F1–F2 with a disulfide bond. As a result of the proteolysis, a hydrophobic peptide of 24 amino acids, the fusion protein, is located at the amino terminal region of F1. This peptide is inserted into the membrane of the host cell and plays an important role in the fusion of NDV. Furthermore, it is believed that processing of the F protein is a critical determinant of NDV pathogenicity. These two surface proteins (HN and F) are the most important targets for the host immune response (Alexander 1997).

NDV strains are classified in three groups according to the severity of the disease they induce. The velogenic strains are highly virulent for chickens of all ages, and they include the viscerotropic forms that cause intestinal hemorrhagic wounds and the neurotropic forms responsible for nervous and respiratory disorders. Chickens infected with velogenic strains develop a severe picture of the disease that generally results in death. A minimum lethal dose with these strains induces death in less than 60 h. The period of incubation is 4–6 days. Mesogenic strains are less virulent and usually produce slight disease, although they can cause death, mainly in young chickens. In some cases, sharp respiratory disorders are present as well as some nervous symptoms. Lentogenic strains are the least virulent, and they only induce a slight respiratory infection. Most of the “live” vaccines in use consist of lentogenic strains, and in some countries mesogenic strains are also used.

NDV infects chickens, turkeys, other birds, and some mammals, including man where it causes conjunctivitis. In the poultry industry, the disease can cause up to 100 % mortality. The surviving chickens do not develop in a normal way; they show low production of eggs and present shells with malformations.

NDV infects both domestic and wild species of birds. Mortality and morbidity rates vary according to the species and in function of the viral strain. Chickens are the most susceptible bird and ducks and geese the least susceptible. The virus is highly transmissible, and transmission can occur by direct contact with feces and respiratory discharges or by contamination of the environment, including food, water, equipment, and human clothing. The greatest potential for spread of the disease is by humans and their equipment. Events such as movement of live birds; contact with other animals; movement of people and equipment; movement of poultry products; airborne, contaminated poultry feed; contaminated water; and contaminated or incompletely inactivated vaccines have been implicated in various epizootics. The virus can survive for long periods in the environment, especially in feces. Migratory birds have been implicated in the primary introduction of the virus, with outbreaks being the result of secondary spread by humans. The virus causes mild conjunctivitis in humans. No known infections have occurred from handling or consuming poultry products.

10.1.3 Economic Impact

ND represents a major limiting factor for increasing poultry production, and it can cause an enormous economic impact at global scale. One can safely say that no other virus comes close in terms of the economic impact on poultry production, and it may represent a bigger drain on the world's economy than any other animal virus. In developed countries, outbreaks of ND and implementation of control measures, including vaccination, are extremely costly for the poultry industry. Countries free of ND are forced to repeat testing to maintain their disease-free status for trade purposes. In developing countries with endemic ND, it has such an economic impact that it becomes an important limiting factor for the development of commercial poultry and trade links. Many developing countries rely on village chickens to supply dietary protein as eggs and meat, especially for women and children, and ND is the most serious constraint for village chicken production throughout the world, particularly in developing countries (Guèye 2002). Continued losses from ND affect the quantity and quality of the food for people on marginal diets.

10.1.4 Current NDV Vaccines: Pros and Cons

Due to the fast dissemination of the disease, probably caused among other things by the intense commercial exchange in the poultry industry, the need for preventive actions became imperative. Thus, the first vaccines for the control of NDV have been developed since the 1940s including formalin-inactivated virus (Schoening et al. 1949; Doyle and Wright 1950), β-propiolactone-inactivated virus (Sullivan et al. 1958), attenuated virus (Bankowski 1957), and live virus (Doll et al. 1951).

Propagation of virus in chicken embryos was being employed since then (Acevedo and Mendoza 1947). Strategies still in use today such as vaccination via the nasal route (Hitchner et al. 1951), in the drinking water (Churchill and Blaxland 1966), or by spray (Rao and Agarwal 1969) were developed around those years. The use of passive immunization (Box et al. 1969) and immunization with a low-virulence viral strain followed by administration with a high-virulence viral strain were developed in the 1960s. Since that time, there have been over 500 publications looking to develop improved vaccines employing conventional technology or to develop new vaccination strategies. To this day, vaccine development against NDV continues to be a very active research area.

ND vaccines are often administered individually to chickens. Catching and handling chickens is an expensive business, and vaccination is often done when chickens are being handled for some other reason. Therefore, methods of mass vaccination are used extensively.

10.1.4.1 Vaccines with Live Virus

These can be divided into two groups: those based on lentogenic or on mesogenic strains, although the latter can only be used as a boost due to their greater virulence but are still permitted in a few areas. These vaccines were mainly developed to establish a controlled infection in each chicken (Allan et al. 1978). Vaccines based on lentogenic strains are administered individually, by the oral, intranasal, or ocular routes, while those based on mesogenic strains generally require inoculation by injury in the membrane of the wing or by intramuscular injection (Alexander 1997). An advantage of the live vaccines is that they can be administered at large scale. The method more popular for administration is by supply in the drinking water, although aerosols are also utilized (Meszsaros 1991). As they are obtained as freeze-dried allantoid fluid from infected embryos, these vaccines are relatively inexpensive and easy to administer; therefore the application at large scale is facilitated. The NDV serotypes share common antigenic properties that allow the use of identical vaccines in different countries. The live ND vaccines are manufactured in a lyophilized form and can be stored at 4 °C for up to a year without losing activity. They stimulate local immunity, and protection can be obtained quickly after application. Likewise, the vaccine can be transmitted to birds not vaccinated from successfully vaccinated birds, taking advantage of the natural dispersion of the virus (Alexander 1997).

There are some disadvantages in the use of live vaccines the most important being that of residual virulence. Most seem capable of causing some disease or slowing growth rates. This is particularly important for virus delivered by spray or aerosol. Respiratory disease and even deaths may result. Some strains of vaccine virus spread naturally between chickens and vaccinated chickens are used to vaccinate other chickens in direct contact. However, dissemination to susceptible groups of birds, especially those where the interval of ages is very large, can cause severe disease, particularly with exacerbating organisms. On the other hand, it has

been known since 1965 that immunity induced by maternal antibodies can diminish the effectiveness of the vaccination with live virus (Box 1965). Finally, it is worth mentioning that these vaccines can be easily inactivated by heat or by chemical agents, and if they are not carefully controlled during their preparation, they can contain contaminating viruses (Gallili and Ben-Nathan 1998). Finally, live vaccines might be involved in the emergence of virulent virus. There is evidence of recombination both in coding and noncoding regions of the NDV genome between vaccine strains and circulating virus resulting in significant genetic change (Zhang et al. 2010). Moreover, the authors also found evidence of homologous recombination between ND viruses of chicken and swine lineages, while the major putative parent is likely to have been derived from the chicken avirulent vaccine lineage. It has been also suggested that viruses of low virulence may mutate to high virulence (Alexander et al. 2012), but this has not been demonstrated and further work is necessary.

10.1.4.2 Vaccines with Inactivated Virus

They are produced from allantoid fluid and treated with ß-propiolactone or formalin to inactivate the virus, which is administered together with a carrier, and they were first developed in the 1950s (Sullivan et al. 1958). The first vaccines with inactivated virus employed aluminum hydroxide carrier and adjuvant, but subsequently, this was substituted by emulsions that contain mineral oil (Gallili and Ben-Nathan 1998). The vaccines with inactivated virus can be administered by intramuscular or subcutaneous injection, and they are more stable than the live vaccines, but their administration is more laborious. As with all the injected vaccines, risks in their application exist, and the toxicity of mineral oils can cause serious problems to the applicator of the vaccine if it is injected accidentally.

Mucosal application of attenuated live virus induces both systemic and local immunity, whereas parenteral immunization with inactivated vaccine generally induces systemic immunity with little local protection (Sharma 1999). A combination of live and inactivated ND vaccine, administered simultaneously, has been shown to provide better protection against virulent NDV and has been successfully used in control programs in areas of intense poultry production (Senne et al. 2004).

10.1.4.3 Recombinant Vaccines

These vaccines have been in development over the last 20 years, and in general they have been reported to confer good protection against NDV. They have employed vectors such as vaccinia and the smallpox virus of the chickens expressing the F and HN proteins, which have conferred protection (Espion et al. 1987; Meulemans et al. 1988; Boursnell et al. 1990a,b; Nishino et al. 1991; Letellier et al. 1991). In addition, other utilized vectors include the herpes virus of turkeys, which, by expressing the F and HN proteins, have protected against the Marek disease and

against NDV when inoculated intravenously (Morgan et al. 1992; Heckert et al. 1996), and the pigeon pox virus (Letellier et al. 1991). Moreover, protection against the NDV has been demonstrated by using the F and HN proteins produced in baculovirus (Nagy et al. 1991; Mori et al. 1994). Likewise, protection against NDV has been reported by intramuscular injection of plasmid DNA carrying the gene of the F or HN proteins (Sakaguchi et al. 1996; Loke et al. 2005). The HN and F genes, expressed in viral vectors, either individually or in combination, have been employed as successful vaccines, which have allowed licensing of two recombinant NDV vaccines in the USA (King 1999). All these recombinant vaccines are efficient and there are several available, but they are not used extensively in the poultry industry because of the high price and by the difficulty for the application at large scale (Seal et al. 2000).

10.1.4.4 Oral Vaccines

Many forms of vaccination have been utilized in chickens, and although the vaccines described before are very efficient, various research teams have emphasized that the best route of vaccination against NDV is by oral administration with vaccines somehow incorporated in the food (Spradbrow and Samuel 1991). This is an option particularly attractive for the security and low cost of vaccination programs. Subunit vaccines administered orally have been generally considered as low risk, since they do not contain any pathogens that could cause adverse effects. Nevertheless, currently there are no subunit vaccines available for oral vaccination at large scale.

Jayawardane et al. (1990) reported total protection of chickens against a velogenic strain of NDV (SL88/1), when the V4 vaccine was supplied using boiled rice as a vehicle. In the same year, Ideris et al. (1990) successfully vaccinated chickens by the oral route with a vaccine sprayed onto food pellets. Subsequently, Spradbrow and Samuel (1991) compared oral vaccination with other routes of vaccination. They observed that after vaccinating chickens with the V4 lentogenic strain, which traditionally is used as a vaccine, antibody titers in serum for the inhibition of hemagglutinin were comparable in chickens vaccinated orally and by the intramuscular and ocular routes and in drinking water. Jayawardane and Spradbrow (1995), trying to explain why the chickens with low titers of antibodies against the NDV presented resistance to the disease after vaccination with the V4 strain, vaccinated chickens by the nasal, ocular, and oral routes. Although formation of IgAs was higher in the intestine of chickens vaccinated by the ocular route, in other places such as the windpipe, the tear fluid, and the serum, the titer of IgAs was higher in chickens vaccinated orally. Rehmani et al. (1995) tested an oral vaccine using lactose-based pellets, which conferred protection against challenge with virulent Newcastle disease virus. Finally Wambura (2009) tested the I-2 vaccine coated on oiled rice in chickens, but only seroconversion was determined. Zhao has experimented with a novel NDV vaccine encapsulated in chitosan

nanoparticles which conferred better protection in chickens compared to the live NDV vaccine strain, LaSota, and the inactivated NDV vaccine (Zhao et al. 2012).

10.1.4.5 Plant-Based ND Vaccines

Based on the previous information, it can be concluded that the optimum route of administration of vaccines against NVD is oral. Nevertheless, in spite of the available vaccines, vaccination to prevent infection of NDV continues to be problematic for two main reasons: the high costs and the difficulty for vaccination at large scale. As vaccines against NVD can be administered with food, the ideal vaccine should be contained in the latter. That is why the possibility arose for this technological development that the food should contain the antigens. In general, conventional vaccines against NDV are very inexpensive (one dose may cost less than 0.01 USD), but this food-based approach may compete successfully from the standpoint of costs against conventional vaccines.

10.2 Description of the System Used to Produce the Antigen

10.2.1 Plants as the Production System for NDV Antigen

Plants are natural bioreactors and are gaining widespread acceptance as a suitable system for the large-scale production of recombinant proteins. The explanation is that plants provide a number of advantages over conventional recombinant systems including low cost, increased safety, and scalable production, among others and, consequently, a wide variety of proteins has been produced in plants which are almost indistinguishable from their native counterparts (Gomez Lim 2011). As molecular farming has come of age, there have been technological developments on many levels, including transfection methods, control of gene expression, expression of multiple proteins, protein targeting, use of different crops as production platforms, and modifications to alter the structural and functional properties of the recombinant product (Thomas et al. 2011). Over the last few years, there has been a continuing commercial development of novel plant-based expression platforms accompanied by significant success in tackling some of the limitations of plants as bioreactors, such as low yields and inconsistent product quality that have limited the approval of plant-derived pharmaceuticals (Gomez Lim 2011). Indeed, one of the most important driving factors has been yield improvement, as product yield has a significant impact on economic feasibility. Strategies to improve the recombinant protein yield in plants include the development of novel promoters, the improvement of protein stability and accumulation, and the improvement of downstream processing technologies. Attention is now shifting from basic research towards

commercial exploitation, and molecular farming is reaching the stage at which it may challenge established production technologies based on bacteria, yeast, and cultured mammalian cells (Davies 2010). There are already several plant-produced proteins on the market including one at large scale employed for diagnostic purposes, but the recent approval of the first plant-derived enzyme for therapy in humans by the FDA (http://www.drugs.com/newdrugs/fda-approves-new-orphan-elelyso-gaucher-3206.html) establishes a credible foothold for more plant-derived pharmaceutical proteins which are reaching the final stages of clinical evaluation, with more in the development pipeline (Yusibov et al. 2011). The low cost of plant-based vaccines make them ideal for large-scale programs in poor countries. Two strategies exist for production of pharmaceutical compounds in plants. One is by the stable transformation of the plant genome, which can be carried out by *Agrobacterium tumefaciens* or via bombardment with microparticles (biolistics). The other involves the use of viruses that infect plants in a natural way, but the foreign gene is inserted only in leaves, which prevents transmission to the progeny.

10.2.2 Previous Attempts to Produce a Plant-Based NDV Vaccine

There have been several attempts to develop a plant-based NDV vaccine (Table 10.1). Berinstein et al. (2005) expressed the F and HN genes in potato and immunized mice intraperitoneally. All mice showed high levels of anti-NDV antibodies in the serum. Mice fed with transgenic potato leaves presented high levels of NDV-specific IgA and lower levels of specific IgG in intestinal tissue. Yang et al. (2007) transformed rice with the F gene, and after immunization of mice, they detected specific antibodies against NDV.

10.2.3 Production of NDV Antigens in Maize for Oral Administration

Several cereals, and in particular maize, have been the system of choice for expression of antigenic proteins since the proteins can be expressed at high levels in the kernel and stored for prolonged periods without excessive deterioration (Streatfield et al. 2003). Dry seeds can be employed as oral vaccines (Lamphear et al. 2002). This was the rationale behind the work of Guerrero-Andrade et al. (2006) who expressed the F protein in maize plants. When the kernels were fed to chickens, it elicited the production of specific antibodies which conferred protection against a lethal viral challenge. This protection was comparable to that conferred by a commercial vaccine. This group has recently produced maize plants containing both HN and F genes, and in similar assays, the edible vaccine protected chickens against a lethal viral

Table 10.1 Newcastle antigens expressed in plants

Newcastle antigen	Plant system	Expression levels	Reference	Protection assays	Model animal
F and HN	Potato	0.3–0.6 μg/mg total leaf protein	Berinstein et al. (2005)	No	Mice
F	Rice		Yang et al. (2007)[a]	No	Mice
F	Corn	1–3 % TSP	Guerrero-Andrade et al. (2006)	Yes	Chickens
HN	Tobacco	0.069 % TSP	Hahn et al. (2007)	No	Chickens
F	Potato	0.25–0.55 μg/100 μg TSP	Yang et al. (2007)	No	Mice
F and HN	Potato	0.3–0.6 μg/mg total leaf protein	Gomez et al. (2008)	No	Mice
HN epitope fused to LTB from *E. coli*	Tobacco chloroplasts	0.5 % TSP	Sim et al. (2009)	No	NA
HN	*Nicotiana benthamiana*	3 μg/mg total leaf protein	Gomez et al. (2009)	No	NA
HN	*Centella asiatica*	3.6–4.0 μg/mg	Lai et al. (2012)	No	NA
HN ectodomain	Tobacco cells	0.2–0.4 % TSP	Lai et al. (2013)	No	Mice

[a]This article was published in Chinese. The information was obtained from the abstract, but it did not mention expression levels
TSP Total soluble proteins

challenge (Gomez-Lim, M.A., unpublished results). Hahn et al. (2007) expressed the HN gene in tobacco plants. Immunized chickens developed slightly high titers of anti-HN serum IgG compared with those immunized with leaves of the wild-type plant which did not induce antibodies, but unfortunately the chickens were not challenged with the virus. Subsequently, Yang et al. (2007) expressed the F protein under the control of two different promoters in transgenic rice. After intraperitoneal immunization of mice with crude extracts, they were able to show that the recombinant protein elicited specific antibodies.

In a follow-up study by Berinstein's group, Gomez et al. (2008) fed adult Balb/c mice with potato leaves for a month and then meticulously studied the immune response at the mucosal level. They found that both immunogens, F and HN, when orally administered, were able to trigger in the gut a host immune response quali- and quantitatively similar to that induced by native NDV. Using a different approach, Sim et al. (2009) fused the heat-labile enterotoxin B subunit from

E. coli to a neutralizing epitope from the HN gene and expressed the fusion protein in *E. coli* and tobacco chloroplasts. The fusion protein was characterized, but no immunological studies were performed. Gomez et al. (2009) tested five different constructs of the HN gene under the control of the Rubisco small subunit promoter in stable and transient assays. The constructs contained the KDEL peptide for retention in the ER. The authors found that the construct harboring the complete HN gene with its own signal peptide, fused to the KDEL retention peptide, yielded the highest HN protein levels regardless of whether transient or stable transformation was performed. Unfortunately, the constructs were not tested in animals.

Lai et al. (2012) expressed the HN gene in a novel system, the medicinal plant *Centella asiatica*. They were able to confirm the presence of the protein. The same group (Lai et al. 2013) transformed BY-2 cells with the ectodomain of the HN gene protein and immunized mice intraperitoneally with purified recombinant protein. The authors detected successful seroconversion in all mice immunized.

10.3 Commercial Potential of a Plant-Based NDV Vaccine

There are few cases of plant-produced proteins where the application is so straightforward, the intended target so well defined, the public perception so amenable to the idea, and the regulatory issues solved as the vaccine against NDV. The use of a plant-derived vaccine for oral administration would provide a new approach for control of NDV in poultry in comparison to conventional vaccines administered by injection, contained in drinking water, or by coarse spray. Such a vaccine would have a number of advantages and a clear commercial application (Table 10.2). Consequently, there has been interest from industry at many levels. Currently there is one US Patent Application (20050048074) that protects the production of the HN gene in plant cells.

Dow AgroSciences has produced an injectable vaccine against NDV based on the HN gene produced in a suspension-cultured tobacco cell line. In a proof-of-concept study, birds were inoculated twice with the vaccine, and over 90 % protection against a lethal challenge was obtained. This vaccine received regulatory approval from the US Department of Agriculture Center for Veterinary Biologics in 2006 and represents the first plant-derived veterinary vaccine approved by the USDA (http://www.thepoultrysite.com/poultrynews/8949/usda-issues-license-for-plant-cell-produced newcastle-disease-vaccine-for-chickens). However, the company is, apparently, not planning to commercialize the vaccine (Yusibov et al. 2011).

Even though the licensed vaccine from Dow is not meant to be administered orally, there exists the technology to develop an oral vaccine. It is likely that many companies in various countries are working actively in this area to develop such a vaccine and administer it locally. Significant hurdles are not apparent from the technological standpoint for a wide-scale application of this technology. The expression levels reported in the literature (Table 10.1) are somewhat low, and it is possible that they could be improved significantly, but as the only challenge study

Table 10.2 Advantages of plant-based oral vaccines against Newcastle

Advantages
Lower cost of vaccination as the antigens are contained in the food
Easy administration
Plant cells provide protection for the antigen in the gut
Plant material (seeds) easy to store and transport without the need for a cold chain
Higher stability of the vaccine (years)
Reduced concerns over contamination with avian pathogens
No dissemination of live virus in the environment (a possible source of lethal strains)
Elimination of syringes and needles

reported so far has shown, these levels are enough to confer protection. Different plant systems have been tested, but cereals, and in particular maize or maybe sorghum, would seem to be the systems of choice as the seeds could be employed without any previous treatment and could be stored for prolonged periods without deterioration (Lamphear et al. 2002; Streatfield et al. 2003).

The licensed vaccine was developed in tobacco cells and administered by injection. Ideally, it would be better to deliver it orally. Commercial vaccines in use today are effective in controlling the disease virus. The disease outbreaks are probably due to problems of vaccine quality or incorrect handling of the vaccines (Gallili and Ben-Nathan 1998). A vaccine contained in plant tissue would not present this type of problem.

One interesting approach that has developed over the years is the use of multivalent vaccines against different diseases. In the case of plant-based vaccines, it would be ideal to combine the expression of different protective antigens in a plant tissue to be delivered orally in chickens. The literature shows a number of publications where this has been tested successfully involving NDV and other diseases (Steel et al. 2008; Vagnozzi et al. 2010). There may be a synergy among the different antigens which enhances the immune reaction. It would be a question of testing the suitability for oral immunization of the other diseases, but in principle, this idea seems very appealing, and it is possible that it has already been tested by some companies.

Another approach that is very attractive is the use of viruslike particles (VLPs) from NDV. VLPs represent a safe, noninfectious strategy to prevent dissemination of diseases such as NDV. They can elicit broadly reactive immune responses that may be equal or superior to antigens employed in current vaccine formulations (Roy and Noad 2009). VLPs, unlike single proteins, have the ability to bind and enter cells using appropriate surface receptors and may be more cost-effective than co-inoculation of multiple single-gene vaccines. VLPs have been obtained from NDV (McGinnes et al. 2010), and foreign epitopes have been inserted and expressed successfully (McGinnes et al. 2011). For that reason, NDV VLPs have been suggested as a convenient platform for development of vaccines for human and animal pathogens (Morrison 2010).

Recently, different research groups have experimented with plant derivatives or extracts to try to enhance the immune response to the NDV vaccines. They have tested garlic (Jafari et al. 2008), extracts of *Momordica cochinchinensis* (Xiao et al. 2009; Rajput et al. 2010), and constituents of *Jatropha curcas* (Abd-Alla

et al. 2009). It will be interesting to see whether they also enhance the response to plant-based oral vaccines against NDV.

Finally, traditional NDV vaccines have been employed in an entirely novel application, the treatment of cancer in humans. The first report that NDV was useful as a cancer treatment was published in 1964. The fact that the virus only causes mild side effects in humans together with its confirmed ability to replicate up to 10,000 times faster in human cancer cells than in normal human cells has led researchers to look more closely at NDV as a possible cancer treatment. There are several reports on the beneficial effect of conventional NDV vaccines and the potential for the virus as a vector for immune therapy and gene therapy of cancer (Csatary et al. 1999; Schirrmacher and Fournier 2009). The effect seems to be via induction of apoptosis of the cancer cells (Fabian et al. 2001). However, randomized controlled trials, enrolling large numbers of people, are required to confirm the results of studies done so far on the use of NDV to treat cancer.

Other potential plant-based products for the veterinary market that may reach the market soon include one against influenza produced by Medicago Inc. and interferon alpha produced in strawberry for treatment of periodontal disease in dogs (METI project) (Yusibov et al. 2011).

10.4 Future Prospects

It comes as no surprise the interest that this technology has generated from a commercial standpoint. In spite of the availability of over 20 different conventional vaccines against NDV, there still is room for innovation and improvement. An oral plant-based vaccine would be welcomed by industry and backyard poultry producers alike, particularly from the standpoint of the considerable reduction in costs of vaccination, together with the fact that there would be no virus dissemination in the environment and likely a reduction in outbreaks. As with many biotechnological developments, this technology has been patented in industrialized countries. Poor countries, which usually have a high Newcastle disease burden (Miguel et al. 2012), often have poor or nonexistent IP protection rules and lack of adequate knowledge and infrastructure to protect and commercialize a biotechnological product. There is an urgent need to develop plant-based low-cost vaccines for poor countries. It is hoped that this technology will eventually help those who need it the most and that the issue of IP does not represent an insurmountable hurdle. Putting the collective benefit ahead of the personal gain will be the key for the full realization of this technology.

References

Abd-Alla HI, Moharram FA, Gaara AH, El-Safty MM (2009) Phytoconstituents of Jatropha curcas L. leaves and their immunomodulatory activity on humoral and cell-mediated immune response in chicks. Z Naturforsch C 64:495–501

Acevedo RA, Mendoza IL (1947) Chicken embryo vaccine against Newcastle disease (pneumoencephalitis). Am J Vet Res 8:91–102

Alexander DJ (1997) Newcastle disease and other avian Paramyxoviridae infections. In: Calnek BW, Barnes HJ, Beard CW, Reid WM, Yoder HW (eds) Diseases of poultry. Iowa State University Press, Iowa

Alexander DJ, Aldous EW, Fuller CM (2012) The long view: a selective review of 40 years of Newcastle disease research. Avian Pathol 41:329–335

Allan WH, Lancaster JE, Toth B (1978) Newcastle disease vaccines: their production and use, FAO Animal Production Series No. 10. FAO, Rome

Bankowski RA (1957) A modified live Newcastle disease virus vaccine. Proc Soc Exp Biol Med 96:114–118

Ben-Nathan B, Drabkin N, Heller D (1981) The effect of starvation on the immune response of chickens. Avian Dis 25:214–217

Berinstein A, Vazquez-Rovere C, Asurmendi S, Gomez E, Zanetti F, Zabal O, Tozzini A, Grand DC, Taboga O, Calamante G, Barrios H, Hopp E, Carrillo E (2005) Mucosal and systemic immunization elicited by Newcastle disease virus (NDV) transgenic plants as antigens. Vaccine 23:5583–5589

Boursnell ME, Green PF, Campbell JI, Deuter A, Peters RW, Tomley FM, Samson AC, Chambers P, Emmerson PT, Binns MM (1990a) Insertion of the fusion gene from Newcastle disease virus into a non-essential region in the terminal repeats of fowlpox virus and demonstration of protective immunity induced by the recombinant. J Gen Virol 71(Pt 3):621–628

Boursnell ME, Green PF, Samson AC, Campbell JI, Deuter A, Peters RW, Millar NS, Emmerson PT, Binns MM (1990b) A recombinant fowlpox virus expressing the hemagglutinin-neuraminidase gene of Newcastle disease virus (NDV) protects chickens against challenge by NDV. Virology 178:297–300

Box PG (1965) The influence of maternal antibody on vaccination against Newcastle disease. Vet Rec 77:246–249

Box PG, Stedman RA, Singleton L (1969) Newcastle disease. I. The use of egg yolk derived antibody for passive immunisation of chickens. J Comp Pathol 79:495–506

Churchill AE, Blaxland JD (1966) Experiments with a live Newcastle disease vaccine administered in the drinking water of chickens. Vet Rec 79:30–35

Csatary LK, Moss RW, Beuth J, Torocsik B, Szeberenyi J, Bakacs T (1999) Beneficial treatment of patients with advanced cancer using a Newcastle disease virus vaccine (MTH-68/H). Anticancer Res 19:635–638

Davies HM (2010) Commercialization of whole-plant systems for biomanufacturing of protein products: evolution and prospects. Plant Biotechnol J 8:845–861

Doll ER, Mc CW, Wallace ME (1951) Susceptibility to Newcastle disease infection of chickens from hens immunized with live virus vaccines. Am J Vet Res 12:232–239

Doyle TM (1927) A hitherto unrecorded disease of fowls due to a filter-passing virus. J Comp Pathol Therap 40:144–169

Doyle TM, Wright EC (1950) An inactivated vaccine against Newcastle disease. Br Vet J 106:139–161

Espion D, de Henau S, Letellier C, Wemers CD, Brasseur R, Young JF, Gross M, Rosenberg M, Meulemans G, Burny A (1987) Expression at the cell surface of native fusion protein of the Newcastle disease virus (NDV) strain Italien from cloned cDNA. Arch Virol 95:79–95

Fabian Z, Torocsik B, Kiss K, Csatary LK, Bodey B, Tigyi J, Csatary C, Szeberenyi J (2001) Induction of apoptosis by a Newcastle disease virus vaccine (MTH-68/H) in PC12 rat phaeochromocytoma cells. Anticancer Res 21:125–135

Gallili GE, Ben-Nathan D (1998) Newcastle disease vaccines. Biotechnol Adv 16:343–366
Gomez Lim MA (2011) Plants as platform for production of pharmaceutical compounds. In: Liong MT (ed) Bioprocess sciences and technology. Nova, New York
Gomez E, Zoth SC, Carrillo E, Roux ME, Berinstein A (2008) Mucosal immunity induced by orally administered transgenic plants. Immunobiology 213:671–675
Gomez E, Zoth SC, Asurmendi S, Rovere CV, Berinstein A (2009) Expression of hemagglutinin-neuraminidase glycoprotein of Newcastle disease virus in agroinfiltrated Nicotiana benthamiana plants. J Biotechnol 144:337–340
Guerrero-Andrade O, Loza-Rubio E, Olivera-Flores T, Fehervari-Bone T, Gomez-Lim MA (2006) Expression of the Newcastle disease virus fusion protein in transgenic maize and immunological studies. Transgenic Res 15:455–463
Guèye EF (2002) Newcastle disease in family poultry: prospects for its control through ethnoveterinary medicine. Livest Res Rural Dev 14:1–13
Hahn BS, Jeon IS, Jung YJ, Kim JB, Park JS, Ha SH, Kim KH, Kim HM, Yang JS, Kim YH (2007) Expression of hemagglutinin-neuraminidase protein of Newcastle disease virus in transgenic tobacco. Plant Biotechnol Rep 1:85–92
Heckert RA, Riva J, Cook S, McMillen J, Schwartz RD (1996) Onset of protective immunity in chicks after vaccination with a recombinant herpesvirus of turkeys vaccine expressing Newcastle disease virus fusion and hemagglutinin-neuraminidase antigens. Avian Dis 40:770–777
Hitchner SB, Reising G, Van Roekel H (1951) Intranasal vaccine: its role in an avian pneumoencephalitis control program. Vet Med 46:21
Hughes CS, Gaskell RM, Jones RC, Bradbury JM, Jordan FT (1989) Effects of certain stress factors on re-excretion of infectious laryngotracheitis virus from latently infected cartier birds. Res Vet Sci 46:274–276
Ideris A, Ibrahim AL, Spradbrow PB (1990) Vaccination of chickens against Newcastle disease with a food pellet vaccine. Avian Pathol 19:371–384
Jafari RA, Jalali MR, Ghorbanpoor M, Saraei SM (2008) Effect of dietary garlic on immune response of broiler chicks to live Newcastle disease vaccine. Pak J Biol Sci 11:1848–1851
Jayawardane GW, Spradbrow PB (1995) Mucosal immunity in chickens vaccinated with the V4 strain of Newcastle disease virus. Vet Microbiol 46:69–77
Jayawardane GW, De Alwis MC, Bandara D (1990) Oral vaccination of chickens against Newcastle disease with V4 vaccine delivered on processed rice grains. Aust Vet J 67:364–366
King DJ (1999) A comparison of the onset of protection induced by Newcastle disease virus strain B1 and, a fowl poxvirus recombinant Newcastle disease vaccine to a viscerotropic velogenic Newcastle disease virus challenge. Avian Dis 43(4):745–755
King DJ (2001) Selection of thermostable Newcastle disease virus progeny from reference and vaccine strains. Avian Dis 45:512–516
Lai KS, Yusoff K, Mahmood M (2012) Heterologous expression of hemagglutinin-neuraminidase protein from Newcastle disease virus strain Af2240 in Centella Asiatica. Acta Biol Cracov Bot 54:142–147
Lai KS, Yusoff K, Mahmood M (2013) Functional ectodomain of the hemagglutinin-neuraminidase protein is expressed in transgenic tobacco cells as a candidate vaccine against Newcastle disease virus. Plant Cell Tissue Organ Cult 112:117–121
Lamphear BJ, Streatfield SJ, Jilka JM, Brooks CA, Barker DK, Turner DD, Delaney DE, Garcia M, Wiggins B, Woodard SL, Hood EE, Tizard IR, Lawhorn B, Howard JA (2002) Delivery of subunit vaccines in maize seed. J Control Release 85:169–180
Letellier C, Burny A, Meulemans G (1991) Construction of a pigeonpox virus recombinant: expression of the Newcastle disease virus (NDV) fusion glycoprotein and protection of chickens against NDV challenge. Arch Virol 118:43–56
Loke CF, Omar AR, Raha AR, Yusoff K (2005) Improved protection from velogenic Newcastle disease virus challenge following multiple immunizations with plasmid DNA encoding for F and HN genes. Vet Immunol Immunopathol 106:259–267

McGinnes LW, Pantua H, Laliberte JP, Gravel KA, Jain S, Morrison TG (2010) Assembly and biological and immunological properties of Newcastle disease virus-like particles. J Virol 84:4513–4523

McGinnes LW, Gravel KA, Finberg RW, Kurt-Jones EA, Massare MJ, Smith G, Schmidt MR, Morrison TG (2011) Assembly and immunological properties of Newcastle disease virus-like particles containing the respiratory syncytial virus F and G proteins. J Virol 85:366–377

Meszaros JF (1983) Control of infectious diseases in large scale poultry breeding and production. Worlds Poult Sci J 39:132–141

Meszsaros H (1991) Aerosol immunization against Newcastle disease. Deut Tierarztl Woch 98:123–126

Meulemans G, Letellier C, Gonze M, Carlier MC, Burny A (1988) Newcastle disease virus F glycoprotein expressed from a recombinant vaccinia virus vector protects chickens against live-virus challenge. Avian Pathol 17:821–827

Miguel E, Grosbois V, Berthouly-Salazar C, Caron A, Cappelle J, Roger F (2012) A meta-analysis of observational epidemiological studies of Newcastle disease in African agro-systems, 1980–2009. Epidemiol Infect 141:1–17

Mohamed MA, Hanson RP (1980) Effect of social stress on Newcastle disease virus (LaSota) infection. Avian Dis 24:908–915

Morgan RW, Gelb J Jr, Schreurs CS, Lutticken D, Rosenberger JK, Sondermeijer PJ (1992) Protection of chickens from Newcastle and Marek's diseases with a recombinant herpes virus of turkeys vaccine expressing the Newcastle disease virus fusion protein. Avian Dis 36:858–870

Mori H, Tawara H, Nakazawa H, Sumida M, Matsubara F, Aoyama S, Iritani Y, Hayashi Y, Kamogawa K (1994) Expression of the Newcastle disease virus (NDV) fusion glycoprotein and vaccination against NDV challenge with a recombinant baculovirus. Avian Dis 38:772–777

Morrison TG (2003) Structure and function of a paramyxovirus fusion protein. Biochim Biophys Acta 1614:73–84

Morrison TG (2010) Newcastle disease virus-like particles as a platform for the development of vaccines for human and agricultural pathogens. Future Virol 5:545–554

Nagy E, Krell PJ, Dulac GC, Derbyshire JB (1991) Vaccination against Newcastle disease with a recombinant baculovirus hemagglutinin-neuraminidase subunit vaccine. Avian Dis 35:585–590

Nishino Y, Niikura M, Suwa T, Onuma M, Gotoh B, Nagai Y, Mikami T (1991) Analysis of the protective effect of the haemagglutinin-neuraminidase protein in Newcastle disease virus infection. J Gen Virol 72(Pt 5):1187–1190

Rajput ZI, Xiao CW, Hu SH, Habib M, Soomro NA (2010) Enhancement of immune responses to infectious bursal disease vaccine by supplement of an extract made from Momordica cochinchinensis (Lour.) Spreng. seeds. Poult Sci 89:1129–1135

Rao SB, Agarwal KK (1969) Studies on vaccination with lentogenic strains of Newcastle disease virus administered through spray and drinking water. Indian Vet J 46:743–749

Rehmani SF, Spradbrow PB, West R (1995) Lactose pellets–a new approach to oral vaccination of village chickens against Newcastle disease. Vet Microbiol 46:47–53

Roy P, Noad R (2009) Virus-like particles as a vaccine delivery system: myths and facts. Adv Exp Med Biol 655:145–158

Sakaguchi M, Nakamura H, Sonoda K, Hamada F, Hirai K (1996) Protection of chickens from Newcastle disease by vaccination with a linear plasmid DNA expressing the F protein of Newcastle disease virus. Vaccine 14:747–752

Schirrmacher V, Fournier P (2009) Newcastle disease virus: a promising vector for viral therapy, immune therapy, and gene therapy of cancer. Methods Mol Biol 542:565–605

Schoening HW, Osteen OL et al (1949) Vaccination against Newcastle disease with formalin-inactivated, commercially produced vaccines. Am J Vet Res 10:176–182

Seal BS, King DJ, Sellers HS (2000) The avian response to Newcastle disease virus. Dev Comp Immunol 24:257–268

Senne DA, King DJ, Kapczynski DR (2004) Control of Newcastle disease by vaccination. Dev Biol 119:165–170

Sharma JM (1999) Introduction to poultry vaccines and immunity. Adv Vet Med 41:481–494

Sim JS, Pak HK, Kim DS, Lee SB, Kim YH, Hahn BS (2009) Expression and characterization of synthetic heat-labile enterotoxin B subunit and hemagglutinin-neuraminidase-neutralizing epitope fusion protein in Escherichia coli and Tobacco Chloroplasts. Plant Mol Biol Rep 27:388–399

Spradbrow PB, Samuel JL (1991) Oral Newcastle disease vaccination with V4 virus in chickens: comparison with other routes. Aust Vet J 68:114–115

Steel J, Burmakina SV, Thomas C, Spackman E, Garcia-Sastre A, Swayne DE, Palese P (2008) A combination in-ovo vaccine for avian influenza virus and Newcastle disease virus. Vaccine 26:522–531

Streatfield SJ, Lane JR, Brooks CA, Barker DK, Poage ML, Mayor JM, Lamphear BJ, Drees CF, Jilka JM, Hood EE, Howard JA (2003) Corn as a production system for human and animal vaccines. Vaccine 21:812–815

Sullivan JF, Gill E, Somer AM (1958) Immune response of chickens to Betapropiolactone-killed Newcastle disease vaccines. Am J Vet Res 19:483–488

Thomas DR, Penney CA, Majumder A, Walmsley AM (2011) Evolution of plant-made pharmaceuticals. Int J Mol Sci 12:3220–3236

Vagnozzi A, Garcia M, Riblet SM, Zavala G (2010) Protection induced by infectious laryngotracheitis virus vaccines alone and combined with Newcastle disease virus and/or infectious bronchitis virus vaccines. Avian Dis 54:1210–1219

Wambura PN (2009) Vaccination of chickens using raw rice coated with novel trehalose nano-organogels containing Newcastle disease (strain I-2) vaccine. Trop Anim Health Prod 41:797–802

Xiao C, Bao G, Hu S (2009) Enhancement of immune responses to Newcastle disease vaccine by a supplement of extract of Momordica cochinchinensis (Lour.) Spreng seeds. Poult Sci 88:2293–2297

Yang ZQ, Liu QQ, Pan ZM, Yu HX, Jiao XA (2007) Expression of the fusion glycoprotein of Newcastle disease virus in transgenic rice and its immunogenicity in mice. Vaccine 25:591–598

Yusibov V, Streatfield SJ, Kushnir N (2011) Clinical development of plant-produced recombinant pharmaceuticals: vaccines, antibodies and beyond. Hum Vaccin 7:313–321

Zhang R, Wang X, Su J, Zhao J, Zhang G (2010) Isolation and analysis of two naturally-occurring multi-recombination Newcastle disease viruses in China. Virus Res 151:45–53

Zhao K, Chen G, Shi XM, Gao TT, Li W, Zhao Y, Zhang FQ, Wu J, Cui X, Wang YF (2012) Preparation and efficacy of a live Newcastle disease virus vaccine encapsulated in chitosan nanoparticles. PLoS One 7:e53314

Chapter 11
An Oral Vaccine for Hepatitis B: Challenges, Setbacks, and Breakthroughs

Celine A. Hayden

11.1 Introduction

11.1.1 Hepatitis B Epidemiology

Hepatitis B virus (HBV) infections continue to be prevalent around the world despite efforts by the WHO to implement immunization programs in all WHO member countries (WHO 2010). Although the commercial vaccine induces seroconversion (>10 mIU/mL) in 85–100 % of individuals (Keating and Noble 2003), the chronic infection rate in western African countries is still ≥8 % and between 5 and 7 % in large parts of Africa, Asia, and Southeast Asia (Ott et al. 2012). An estimated 240 million individuals harbor chronic infections of the virus and as a result, 15–25 % will die prematurely due to cirrhosis of the liver or liver cancer (CDC 2006). In the USA, approximately 730,000 individuals are chronically infected (Wasley et al. 2010) despite pervasive neonate vaccination programs.

The number of chronic carriers in the USA is at first perplexing given the efficacy of the commercially available parenteral vaccine but, upon deeper analysis, can be explained. HBV is highly resistant to environmental degradation and can survive on surfaces up to 7 days (Bond et al. 1977). It is transmitted readily via the blood or bodily fluids of a chronic carrier, most of whom do not display outward symptoms of the disease (CDC 2006). Given the virus' environmental resilience and ease of transmission both systemically and mucosally, exposure to infective virus is relatively commonplace in subpopulations with high rates of chronic infection. Of note, mothers who are chronic carriers transmit their infection to their newborns at very high frequencies if newborns are not vaccinated or treated with immunoglobulin therapy (Isaacs et al. 2011). Neonates are most susceptible to

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the virus, progressing to chronic infection in 75–90 % of infected infants (under 6 months). Children under 5 years of age develop chronic infections in 30 % of cases and adults in 5–10 % of cases (Edmunds et al. 1993; Hyams 1995; Isaacs et al. 2011; McMahon et al. 1985). As of 2007, 60 % of US neonates were administered a hepatitis B vaccine birth dose, up from 30 % in 1999, but well below the Healthy People 2020 target of 85 % (Zhao et al. 2011). Increasing neonate vaccination in the USA and worldwide will undoubtedly help reduce the chronic disease burden, but may not be sufficient to eradicate the disease.

In addition to neonate susceptibility to HBV and its tenacious virulence, other factors perpetuate the infection-transmission cycle. Many subpopulations in the USA have low rates of vaccination. Individuals who have been formerly incarcerated and those who are at high risk of exposure to HBV (e.g., hemophiliacs, injection drug users, men who have sex with men, patients with HIV-positive serum) all showed evidence of vaccination rates of less than 60 % (Lu et al. 2011; Lum et al. 2003; MacKellar et al. 2001; Nyamathi et al. 2012).

For segments of the population that are at higher risk of hepatitis B infection, such as healthcare workers, persons engaging in high-risk sexual activity, HIV patients, dialysis patients, diabetics, and children at risk for cystic fibrosis-associated liver disease, full vaccination schedules and additional boosters are recommended by the CDC until seroconversion is achieved (CDC 2006, 2011; Schillie et al. 2012; Shapiro et al. 2013). Unfortunately, many of these individuals are poor immunologic responders to the commercial vaccine. Seroconversion following vaccination with the hepatitis B surface antigen (HBsAg) is considered complete once anti-HBsAg titers reach $\geq$10 mIU/mL. While this level is achieved in 85–100 % of the general population, titers reach acceptable levels in only 64 % of dialysis patients (Chaves et al. 2011), 41–60 % of individuals positive for HIV (Landrum et al. 2012; Laurence 2005), 35 % of individuals with irritable bowel disease (Vida Pérez et al. 2009), 50 % of celiac disease patients (Ahishali et al. 2008; Leonardi et al. 2009), 38 % of obese individuals (Roome et al. 1993), 30–58 % of the elderly (Roome et al. 1993; Tohme et al. 2011; Williams et al. 2012), and 57 % of those with chronic kidney disease (Zitt et al. 2011). There is some evidence that other routes of vaccine administration may be effective in eliciting responses in nonresponders, such as by intradermal administration in healthcare workers (Levitz et al. 1995; Nagafuchi et al. 1991; Roukens et al. 2010) and in children with celiac disease (Leonardi et al. 2012). Unfortunately the present commercial vaccines are not approved for intradermal administration and further resources will need to be allocated to pursue this healthcare model. Many of the other nonresponding or poorly responding populations may also benefit from different administration routes, such as intradermal, sublingual, or oral routes, since they may successfully stimulate different immunologic tissues and confer greater protection. Novel adjuvant-formulated vaccines also show promise in poor responders, but, as yet, none have gained licensure following review by the Food and Drug Administration (FDA).

Education campaigns are also likely to improve the outlook for reducing HBV infections. Recent hepatitis B outbreaks in assisted living and long-term care

facilities arose due to sharing of blood glucose monitoring equipment (Tohme et al. 2011; Williams et al. 2012) and could have been avoided with training of staff and patients. Education, along with additional boosting doses, should contain future outbreaks to a minimum.

In the USA, emigrating populations carry a higher burden of chronic infections than the general population (Hur et al. 2012), likely due to inaccessibility of healthcare in originating countries. This translates to higher rates of chronic infection in Asian-American, Pacific Islander, and Asian/Latino communities, which range from 8 to 17 % (Hur et al. 2012; Lin et al. 2009). The problem, therefore, is truly a global one and can only be addressed when all countries can adopt adequate vaccination strategies. Unfortunately, the cost of vaccination activities in fifty low-resource countries was projected to be US $27 per infant with a US $5 per infant shortfall in funding in 2010 (Kamara et al. 2012). Clearly a more cost-effective approach is needed to combat vaccine-preventable diseases worldwide.

11.2 The Ideal Vaccine

The gold standard for a vaccine is one that is low cost, safe, highly effective in all segments of the population, easily administered, stable at ambient temperatures, easily transported and stored, and does not produce large amounts of hazardous waste. Presently, there is no vaccine that meets all of these standards, and it is clear that not all criteria must be met in order to eradicate disease. The smallpox virus was eradicated using a highly effective parenteral vaccine that required a cold chain. The cold chain requirement significantly increased logistical issues surrounding delivery but was not, in the end, insurmountable given enough resources and determination. Nonetheless, the more attributes we can impart to next-generation vaccines, the more likely they will be to successfully contain and eliminate disease worldwide.

11.2.1 Currently Available Vaccines

The most widely commercialized parenteral hepatitis B vaccines consist of the small hepatitis B surface antigen (HBsAg) produced as a recombinant protein in a variety of yeast strains. A full course adult vaccination consists of three 10 or 20 μg doses administered over a 6–12-month timeframe. Recombivax® is marketed by Merck (1998), while Engerix-B® (GSK 1989) and Twinrix® (GSK 2001) are marketed by GlaxoSmithKlein. Sanofi-Aventis has also joined the market, producing Shanvac B® through its subsidiary, Shantha Biotech (Shantha 2009), and Sanofi-Pasteur MSD produces HBvaxPRO® (Sanofi-Pasteur 2011) for the European market. Other manufacturers also market recombinant HBsAg such as the Serum Institute of India, Ltd. in India producing Gene Vac-B® (Serum Institute

of India 2009) and Scott Cassara in Argentina producing AgB® (Scott Cassara 2009). These commercialized products seem to be highly effective, inducing seroconversion in >90 % of test populations (Lepetic et al. 2003; Rustgi et al. 1995; Shivananda et al. 2006; Tielemans et al. 2011; Velu et al. 2007).

Several subtypes of hepatitis B occur worldwide, and the presence of the “a” determinant region in the vaccine antigen, a highly antigenic site from amino acid position 139–147, seems essential to targeting of all common subtypes. There have been reports of G to R mutations at position 145 in the “a” determinant (Carman et al. 1990; Yamamoto et al. 1994), but these reports are rare and the G to R mutation seems to strongly compromise infection rates. In chimpanzees, the G to R mutant was unable to establish an infection in one individual and in the other test subject caused a much delayed infection that co-occurred with reversion to the wild-type HBsAg sequence (Kamili et al. 2008). There has been some suggestion that a hepatitis B vaccine should include a G to R mutant HBsAg molecule, but to date no products have incorporated this variant into their commercialized vaccine.

In order to produce more robust antibody titers in poor responders, naturally occurring larger forms of the antigen have been tested for their immunogenicity. Middle (M) and large (L) forms of HBsAg are composed of the small HBsAg sequence in addition to an N-terminal preS2 sequence, or the preS1 and preS2 sequences, respectively. There is evidence that the M and L forms are more immunogenic than the small form (Milich et al. 1986, 1985a, b), and Bio-Technology General® Ltd has produced L-HBsAg in Chinese hamster ovary cells and marketed it in Israel (BioTechnology General 2001). This vaccine induces earlier seroprotective titers when compared to Engerix-B (Shapira et al. 2001) and confers seroprotective titers in all tested newborns after only two doses (Madaliński et al. 2004). This third-generation hepatitis B vaccine also shows improved seroconversion in poor responders with end-stage renal disease (Weinstein et al. 2004) and may be a good option for nonresponders and reduced dosing of infants and adults alike.

11.3 The Case for an Oral Vaccine

Despite the reported efficacy of traditional hepatitis B vaccines and the improvements gained with third-generation vaccines, there is still a pressing need for an improved vaccine on several fronts. Significant encumbrances of the parenteral vaccine system include a high cost per dose, the pervasiveness of needle aversion in society, the requirement for trained medical personnel for administration, the need for waste and disposal management, and the need for boosting doses throughout one’s lifetime. Also, as mentioned above, nonresponders and poor responders are still at risk of infection after parenteral immunization. An oral vaccine could potentially circumvent all of these hurdles and, with some systems, offer additional benefits such as elimination of the cold chain. All of these factors are especially

pertinent in resource-poor countries where the highest burden of hepatitis B infection exists.

Encouragingly, the financing of vaccination programs for 50 low-resource countries has been steadily increasing over the last ten years, from $6 per infant in 2001 to a projected $22 per infant in 2010 (Kamara et al. 2012). Governments are contributing $8 per infant, while the remaining $14 is funded by the Global Alliance for Vaccines and Immunizations (GAVI) and various other funding sources. Despite the increases in funding, however, there is still a projected $5 shortfall per infant in funding. This is clearly an unsustainable situation, and steps must be taken to reduce the cost of vaccine delivery so that individual nations can afford to subsidize their own vaccination programs. By far the largest cost contributor is Vaccine Supply and Logistics, accounting for 62 % of immunization expenditures (Kamara et al. 2012). Early cost models applied to low-resource countries have assumed a cost of US$1 per dose (Hall et al. 1993), and recent reductions in price reported by GAVI have set the price of a monovalent hepatitis B dose at US $0.18 (GAVI Alliance 2012). But it is not clear whether these gains in price reduction have resulted from increased subsidies, companies in emerging markets providing more cost-effective products, or economies of scale. In contrast, an oral vaccine from plants could be supplied at a much lower cost without subsidies, incurring significant savings (Howard and Hood 2007). An oral vaccine would also promote savings in Service Delivery, which accounts for 23 % of the cost of immunization (Kamara et al. 2012), since no highly skilled personnel, needles, or hazardous waste disposal system would be required for administration and waste management.

An oral vaccine is a very attractive option not only in terms of cost savings but also in terms of patient preference. A fear of needles is manifested in 45 % of adults and 62 % of children and is the primary factor in vaccine noncompliance (Taddio et al. 2012). An oral vaccine could alleviate noncompliance and stress in this significant segment of the population. Incomplete administration of the full three-dose hepatitis B vaccination series is an issue not only in the general population (Dannetun et al. 2006; Lu et al. 2011) but also in healthcare worker populations. Among Swedish healthcare workers, 79 % received at least one dose, but only 40 % completed the three-dose series (Dannetun et al. 2006), among US at-risk healthcare workers, 75 % completed the three-dose series (Simard et al. 2007), and among healthcare workers in Burkina Faso, only 48 % received at least one dose and a paltry 11 % received the full vaccination series (Ouédraogo et al. 2013). In addition to cost and inconvenience, needle aversion may explain some of the observed noncompliance.

These noncompliance factors are becoming more pertinent as evidence mounts that additional booster doses beyond the three-dose regime are required for various at-risk populations. Poor responders can reach seroconversion but must undergo one or more supplementary boosts before reaching titers >10 mIU/mL. Hepatitis B outbreaks in assisted living and long-term care facilities (Tohme et al. 2011; Williams et al. 2012) have necessitated the administration of booster vaccinations for residents, many of whom are poor responders. While the parenteral vaccine is shown to produce protective titers 5–23 years after initial postnatal doses (Behre

et al. 2012; Poovorawan et al. 2012; Wu et al. 2012), not all individuals maintain protective titers for such long periods of time and not all low-titer individuals (<10 mIU/mL) regain seroprotective levels with a single boosting dose. In one study, 30 % of individuals who had lost seroprotective titers after 15 years did not mount an anamnestic response upon boosting, indicating that they require further boost doses to reach seroprotective levels (Chaves et al. 2012). It is recommended that all poorly responding and at-risk populations should receive the hepatitis B vaccine and booster doses, as outlined by the CDC (2006), which would be greatly facilitated by an oral vaccine.

As with other orally administered drugs, oral vaccines could be obtained directly from a pharmacy, eliminating the need for a doctor's office visit and the associated costs. This would significantly increase convenience for obtaining the drug, decrease distribution cost, and potentially improve compliance of boosting regimes. Safety issues associated with injected vaccines such as incomplete sterilization of needles and blood contamination from improper needle disposal would become irrelevant in an oral system.

With lyophilized or bioencapsulated products, the vaccine could also be stored in a dry form at ambient temperatures, eliminating reliance on preservatives and cold storage distribution systems. Storage under ambient conditions would represent a significant advantage in areas of the world where cold storage is a significant logistical hurdle, such as remote villages or where electricity is not reliably available. Furthermore, preservatives would not be required for lyophilized or dried products, which would simplify distribution worldwide. Presently, there is a United Nations-backed initiative to limit the production, importation, and exportation of mercury-containing products, including ethylmercury found in the vaccine preservative thimerosal (Durrheim and Poland 2013). Because the parenteral vaccine is in a liquid form, it requires use of a preservative in multidose vials, vials which are more cost-effective than single-dose alternatives. An oral vaccine would offer a mercury-free option and reduce the cost of the vaccine, providing a very attractive vaccine alternative to resource-poor countries.

Lastly, an oral vaccine could confer distinct advantages in terms of mucosal immunity that would be complementary to the systemic immunological response induced by the parenteral vaccine. At least 70 % of all Ig in mammals derives from IgA produced at mucosal sites (Macpherson et al. 2008), and therefore these sites are large factories for IgA deployment and immunologic protection. An oral vaccine could activate antigen presenting cells in the oral cavity and the gut, mucosal sites which have been shown to disseminate IgA antibodies throughout the body, including the small intestine, the colon, the stomach, the respiratory tract, the reproductive tract, and the blood (Czerkinsky and Holmgren 2012). It seems that only a combination of oral and sublingual routes of administration can produce such widespread distribution of IgA antibodies throughout the body, while nasal, rectal, vaginal, and transdermal routes of immunization produce much more localized antibody responses.

11.3.1 Oral Vaccine Systems Targeting Hepatitis B

Since the licensing of the hepatitis B parenteral vaccine in 1989, many groups have attempted to produce a mucosal subunit vaccine against HBV. Each system has had to contend with issues of encapsulation, increased dosing, and cost of processing. In order for the antigen to stimulate antigen presenting cells (APCs) in the gut, a sufficiently protected dose must be ingested. Conditions in the gastrointestinal (GI) tract lead to protein cleavage and denaturation therefore some form of encapsulation is required for the maintenance of antigen integrity. Bioencapsulation and the use of nanoparticles are the two most popular approaches to protecting the antigen, with significant degrees of success. Encapsulation of the antigen is necessary but not sufficient to induce an immunologic response. For a robust immune response, a large enough dose must be ingested so that a critical mass of intact antigens can reach the APCs of the Peyer's patches. In order to induce immunologic responses, the oral dose is typically 100–1,000 times the injected dose (Hayden et al. 2012b); (unpublished data) which increases cost significantly if the antigen is purified and encapsulated. Therefore, in order for an oral delivery method to be cost-effective, an extremely low cost of production of HBsAg is critical to its success.

The various approaches to develop an oral vaccine can be broadly classified into plant-based and non-plant-based systems. The earliest attempts were non-plant-based and involved oral administration of adenoviruses Ad4 and Ad7 expressing HBsAg (Lubeck et al. 1989). The adenoviruses were chosen for their ability to infect enteric tissues, but antibody responses were relatively low in a chimpanzee model. Furthermore, the system relied on production of the adenoviruses in a human carcinoma cell line, A549. Human cell lines are not ideal systems for production as they are susceptible to infection by human pathogens, which can then reside in the final product. Concurrently, another research group expressed HBsAg epitopes in *Salmonella* and administered the material either intramuscularly (i.m.) or orally. Disappointingly, the orally delivered material produced titers that were ten times lower than those produced from i.m. delivery (Wu et al. 1989).

Since then, *Salmonella* has been used as a DNA vaccine vector with some success when fed to mice. These DNA vaccines appear to induce stronger cytotoxic T lymphocyte responses, but weaker IgG antibody responses than parenteral vaccines (Woo et al. 2001). The authors suggest that DNA vaccines in *Salmonella* could therefore be used therapeutically, rather than as a preventative vaccine, and subsequent studies in an HBsAg-expressing transgenic mouse model have supported these conclusions (Zheng et al. 2002). DNA vaccines have also been administered orally with biodegradable microparticles and shown immunologic activity in mice (He et al. 2005), but as yet, it is not clear how they compare to commercially available parenteral vaccines.

Encapsulation of HBsAg epitopes or full-length antigen using various types of small particles such as poly D,L-lactide co-glycolide (PLG), alginate-coated chitosan, bilosomes, and copolymers has been a promising approach for induction of immune responses in mice (Borges et al. 2007; Gupta et al. 2007; Jain et al. 2010; Rajkannan

et al. 2006; Shukla et al. 2008). The primary drawback to these methods is the added cost of production. The hepatitis B vaccine is already highly subsidized in poorer countries, and an inflated cost would hamper its distribution worldwide. Furthermore, it is unclear whether these nanoparticles will have acceptable safety profiles upon ingestion.

Most recently, *Lactococcus lactis*, a bacterium used to produce yogurt products, has been engineered to express preS HBsAg sequences (Zhang et al. 2011). This bacterium has a long history of use in dairy fermentation products and therefore has a very strong consumption safety profile. However, the possibility of lateral gene transfer of rHBsAg from the ingested *Lactococcus* to resident gut bacteria may represent a serious roadblock to commercialization of this product. Furthermore, although *Lactococcus* bacterial pastes induced IgA responses in intestinal samples and IgG responses in serum, additional work is required to determine the immunologic efficacy of these sequences relative to the parenteral vaccine.

Plant-produced hepatitis B vaccines have sustained interest in the research community as evidenced by the steady stream of plant host species adopted for the production of recombinant HBsAg (see Table 11.1). They have produced strong immunologic responses in mice and have shown promise in human clinical trials. Several technical challenges have been overcome, but a few must still be addressed before commercialization can be achieved.

11.4 Technical Challenges, Setbacks, and Breakthroughs

11.4.1 Expression Level of HBsAg in Edible Tissues and Response in Mice

Undoubtedly the most significant challenge in producing an oral HBsAg vaccine in plants has been the ability to accumulate sufficiently high levels of antigen. Large amounts of orally delivered HBsAg are required to ensure that antigen reaches APCs in the gut to induce an immunologic response. Oral doses may require 100 times the parenteral dose to be effective. Where most vaccines on the market are administered at 10–20 μg adult doses, this translates to an oral dose of 1 mg, levels that would be prohibitively expensive if using a purified, nanoencapsulated product. These high oral dosing requirements define minimal concentrations of HBsAg needed to produce a realistic commercialization scenario.

HBsAg is a membrane-bound protein, a class of proteins which can be difficult to express at high concentrations in heterologous systems (Grisshammer 2006). Early attempts at producing the antigen in plants resulted in very low HBsAg concentrations in lettuce, lupin, and tomatillo (Fig. 11.1) and weak immunogenic responses in mice (Gao et al. 2003; Kapusta et al. 1999). Production of the antigen in potato has been more promising. When mice were fed potato tubers with 1.1 μg HBsAg/g of tuber + 10 μg cholera toxin (CT) as a primary vaccine and boosted with an injected dose of yeast recombinant HBsAg, mice achieved antibody titers of 1,679 mIU/mL

Table 11.1 Production of HBsAg in plants and oral immunogenicity

Plant species	Antigen form	Formulation	HBsAg expression level	Oral immunogenicity	Citation
Lupin	HBsAg	Raw callus fed orally	0.15 μg/g FW	Weakly immunogenic in mice	Kapusta et al. (1999)
Lettuce	HBsAg	Leaves fed orally	0.006 μg/g FW	Weakly immunogenic in humans	Kapusta et al. (1999)
Potato	HBsAg	Raw tuber fed orally + cholera toxin (CT)	8.5 μg/g FW	Mice reach 3,300 mIU/mL	Kong et al. (2001)
Tomatillo	HBsAg	Fruit tissue fed orally	0.3 μg/g FW	Weakly immunogenic in mice	Gao et al. (2003)
Potato	Middle HBsAg (preS2 + S)	Potato extract + 10μg CT or raw potato tuber + CT	0.09 % TSP	Immunogenic in mice	Joung et al. (2004), Youm et al. (2007)
Banana	HBsAg	None tested	0.02 μg/g FW	Not tested	Sunil Kumar et al. (2005)
Potato	HBsAg	Raw tuber fed orally	8.5 μg/g FW	Immunogenic in 62.5 % of human volunteers	Thanavala et al. (2005)
Tomato	TBI-HBsAg	Lyophilized fruit orally fed	50–70 μg/g DW	Immunogenic in mice	Salyaev et al. (2009), Shchelkunov et al. (2006)
Tomato	Large HBsAg (preS1 + preS2 + S)	None tested	0.52 μg/g FW	Not tested	Lou et al. (2007)
Lettuce	HBsAg	Lyophilized leaves fed orally at low dose (100 ng)	60 μg/g FW	Weakly immunogenic in mice	Pniewski et al. (2011)
Maize	HBsAg	Maize germ fed orally	166 μg/g DW	Immunogenic in mice	Hayden et al. (2012b)

FW fresh weight, *DW* dry weight, *TSP* total soluble protein

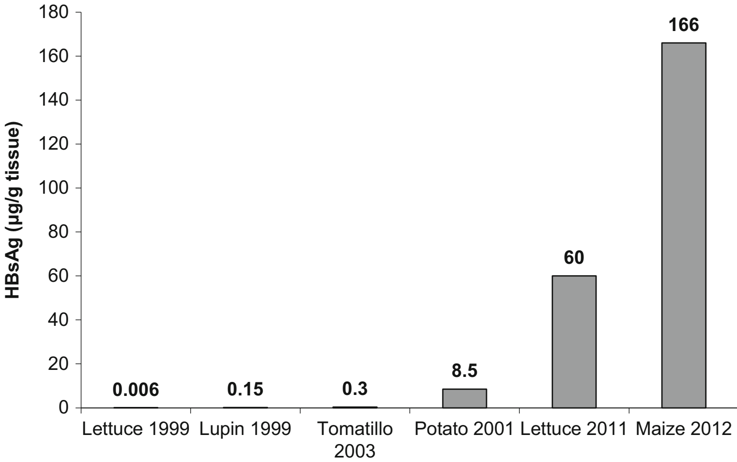

Fig. 11.1 Gains in HBsAg accumulation in various plant species over 13 years. See Table 11.1 for referenced literature

(Richter et al. 2000). Encouragingly, when mice were fed a tuber with a higher concentration of antigen (8.5 µg/g + CT) with a similar vaccination regime, antibody levels increased twofold to a maximum of 3,300 mIU/mL (Kong et al. 2001), indicating that antigen concentration for oral delivery is a limiting factor to immunologic response. Greater accumulation of antigen has since been achieved in maize germ (166 µg/g dry weight), and mice fed this material as a booster produced robust systemic and mucosal antibody responses, even in the absence of an adjuvant (Hayden et al. 2012b). Compared to potato material + CT used as an oral booster (Kong et al. 2001), the maize material produced a threefold higher increase in total serum antibody (Fig. 11.2). Furthermore, maize breeding strategies, such as production of hybrid grain, can further increase HBsAg expression in maize material (Hayden et al. 2012b). More highly expressing constructs have been developed for maize germ expression and should achieve additional gains in expression following a hybrid breeding program (Hayden et al. 2012a). An incomplete backcross and hybrid breeding program has already produced material that expresses upward of 500 µg/g (unpublished data) and therefore shows exceptional promise as a candidate oral vaccine.

Nicotiana benthamiana, using the transient MagnICON viral vector system, has averaged 295 µg/g of fresh weight (Huang et al. 2008), but this system is designed for purification and injection of the antigen, rather than oral delivery. *Nicotiana* species typically produce noxious secondary compounds that are not compatible with an oral delivery system and therefore are not good candidates for oral vaccines in an unpurified form. While purification is an option, it adds substantial cost and diminishes the advantages of the plant-based approach.

Determination of expression level and comparison between plant host systems has been somewhat confounded by the use of different extraction buffers and extraction conditions used by various research groups, which can greatly affect

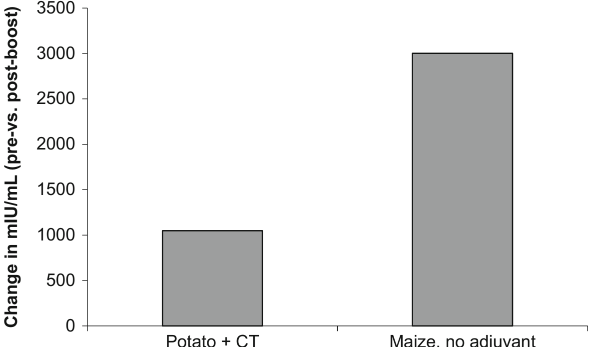

Fig. 11.2 Murine immunologic response to plant-derived HBsAg. Mice were injected with 0.5 μg yeast-derived rHBsAg and boosted with three oral doses of raw plant material expressing HBsAg. Maize was fed at a concentration of 166 μg HBsAg/g of grain and potato was fed at a concentration of 8.5 μg HBsAg/g of potato tuber. Adapted from (Kong et al. 2001) and (Hayden et al. 2012b)

the antigen extraction efficiency and, therefore, the calculated antigen concentration. In addition, a popular standard kit for HBsAg detection, Abbott's Auszyme monoclonal kit, was discontinued from their catalogue several years ago. Therefore, reported concentrations should be used as a guide to assess whether an oral vaccine has the potential to elicit an immunogenic response, and not as a definitive benchmark for whether oral material will elicit a response.

11.4.2 Structural Equivalence

Correct intramolecular disulfide linkages are required to form proper oligomeric structures (Mangold et al. 1997, 1995) and seem to be required for induction of a humoral response (Vyas et al. 1972). Several studies have shown that plant-produced HBsAg can form virus-like particles (VLPs) and oligomeric structures that mimic the conformation of the native virus particle, indicative of correct disulfide bond formation in potato, tobacco, tomato, and maize (Hayden et al. 2012b; Kong et al. 2001; Lou et al. 2007; Mason et al. 1992; Sojikul et al. 2003). It is therefore thought that plants are suitable biofactories for the production of immunogenic HBsAg vaccines.

11.4.3 Immunologic Response in Human Volunteers

An early attempt to induce immunologic responses in human clinical trials was conducted with lettuce tissue expressing HBsAg (Kapusta et al. 1999). Human volunteers were fed two 150–200 g doses of lettuce leaves which contained between 0.15 and 1.0 μg of HBsAg. Volunteers exhibited transient and weak immunologic responses, with serum titers reaching a peak antibody titer between

13 and 18 mIU/mL in two of the three volunteers and quickly dropping below 5 mIU/mL 4 weeks after the second dose.

A second human clinical trial has shown much more promising results using potato tubers as an oral vaccine medium (Thanavala et al. 2005). The potato material was tested as a candidate booster and all volunteers in this study had previously received a hepatitis B parenteral vaccine, displaying titers >10 mIU/mL and <115 mIU/mL at the onset of the study. Volunteers were fed 100–110 g of raw potato tuber in three doses at two week intervals. Each dose contained approximately 850 μg of HBsAg, and this antigen concentration was enough to induce an increase in serum antibody titer in 62.5 % of volunteers (10 out of 16), with 25 % of subjects reaching titers in excess of 1,000 mIU/mL.

This experiment illustrates an important proof of concept as it unequivocally demonstrates the potential of a plant-based HBsAg subunit vaccine as a booster in humans. Clearly, further improvements are needed to produce a commercial product as effective as the parenteral vaccine, but it is likely that a more highly concentrated material or material formulated with an effective mucosal adjuvant could induce a stronger immune response. These improvements may also reduce the number of required doses, which would be ideal for a commercialized product.

11.4.4 Stability, Integrity, and Immunogenicity of HBsAg Through the GI Tract

Oral vaccine strategies are predicated upon the assumption that the antigen is stable and can survive adverse conditions in the GI tract and stimulate APCs in Peyer's patches of the gut. Often overlooked are the APCs present in sublingual tissues, which can complement the immunologic response induced in the gut (Czerkinsky and Holmgren 2012). Therefore, an ideal oral delivery system may seek to stimulate APCs not only in the gut but also in the mouth. Broad activation of APCs could be facilitated by simply administering antigens in wafer form, as opposed to pill form, to increase oral residence time.

From the moment antigens enter the mouth, they must weather protease attack in saliva, the stomach, and the intestines, and survive the low pH of the stomach. Bioencapsulation of proteins in plant tissue can afford protection, allowing the antigen to reach target tissues (Bailey 2000). Some plant tissues may be more effective at delivering intact antigen to the gut by virtue of their cellular environments. For example, maize seeds are replete with protease inhibitors and carbohydrates, conditions that are conducive to maintaining antigen integrity and stability (Arakawa and Timasheff 1982; Boisen 1983).

One complicating factor in using fresh plant tissue for encapsulation is the large fluctuations in antigen concentration over different stages of plant development. In tomato fruits, recombinant antigen concentrations vary considerably, accumulating 100-fold greater concentrations of HBsAg in mature fruit compared to small or

medium fruit (Lou et al. 2007). These fruits must be harvested at an exact stage of maturity to optimize antigen concentration which complicates timing of oral administration. Additionally, mature tomato fruits do not store for long periods of time, and it is not clear, once picked, how the antigen concentration will be affected. Lyophilization can improve the stability of the antigen and concentrate it within the tissue, but this added step does incur additional costs to the process. Unlike fresh tissues, cereal grains, such as rice or maize, provide a dry environment which promotes stability in the final mature form of the grain. Maize grain can be stored for extended periods of time between −20 °C and 55 °C with little effect on HBsAg concentration (Hayden et al. 2012a), a significant advantage over fresh tissue production systems (see Storage and Distribution section, below).

11.4.5 Palatability and Digestibility

When considering a plant production system, serious contenders to commercialization must take into account issues of palatability and digestibility of the plant tissue. Without both good palatability and digestibility, adoption by the market will be difficult. Potato tuber has been a promising plant production system in terms of HBsAg expression levels and immunologic responses in mice and humans, but the vaccine must be administered in 100 g doses of raw tuber to induce a response in human volunteers. Raw potato is not easily digestible (Martinez-Puig et al. 2003) and only becomes highly digestible after cooking, which reduces immunologic responses more than 20-fold, essentially inactivating the antigen (Kong et al. 2001). This system may still be usable if concentrations of HBsAg can be increased such that a very small amount of plant material can be ingested for a full dose. Fruits and grains are more palatable and digestible than raw potato tubers and are much better candidates for commercialization.

11.4.6 Cost

The cost of producing a vaccine dose is one of the determining factors of commercialization potential. Presently, a pediatric injected vaccine dose can be obtained for US$21–24 (CDC 2013). Cost analyses of producing recombinant proteins in plant systems are scarce and usually include a protein purification step. The cost of these systems range from US$0.1/g to $43/g (Evangelista et al. 2008; Hood et al. 2002; Nandi et al. 2005). When purification of the antigen can be eliminated, as is the case with orally delivered edible plant vaccines, the cost is significantly reduced and a dose can be produced in maize grain for less than US$0.01 (Howard and Hood 2007). This assumes the grain expresses 100 μg HBsAg per g of grain and that a dose is less than 10 mg of active HBsAg. Maize grain, therefore, is a strong contender for oral vaccine commercialization and could be a game changer in low-income countries.

It remains to be seen whether other plant systems can provide such favorable economics. Disadvantages of other systems in this arena include lower expression levels than the maize system, and additional processing costs for lyophilization or alternative preservation methods, which would increase the overall cost of production.

11.4.7 Storage and Distribution

Constraints surrounding storage and distribution of oral vaccines are primarily felt in resource-poor countries where refrigeration is not always available or, when available, not reliable. Unlike many other proteins, HBsAg is fairly stable at ambient temperatures, but does lose antigenicity when exposed to 45 °C for 1 week or 37 °C for 1 month (Hipgrave et al. 2006; Otto et al. 1999; Van Damme et al. 1992). Proteins expressed in cereal grains can remain stable for several years at ambient temperatures (Fischer et al. 2004; Lamphear et al. 2002), and HBsAg expressed in maize can survive freezing and extreme ambient heat (55 °C) for at least 1 month (Hayden et al. 2012a). The exceptional stability profile over time and a range of temperatures of maize-based HBsAg is a game changer for populations in remote areas and in regions with absent or weak cold chain infrastructure. In addition, these attributes are particularly salient for stockpiling of vaccines. In the unlikely event of an HBsAg vaccine shortage, an oral plant-based vaccine could be deployed on short notice to large segments of the population by minimally trained personnel. Presumably lyophilized tissue could undergo similar storage and distribution conditions, but this has yet to be tested for plant material.

Under nonemergency conditions, oral doses could be distributed as wafers through a pharmacy or local health clinic. This is a distinct advantage over parenteral vaccines which must be administered by a trained health professional who must then dispose of biological waste and needles. Assuming a 1 mg dose, HBsAg would need to be present at levels of 3,000 μg/g to be administered in a 300 mg pill. Although these levels have not yet been achieved in plant systems, breeding efforts in maize are in progress to reach these levels (unpublished data). Alternatively, an edible wafer (or multiple wafers) weighing between 3 and 10 g and containing 100 μg HBsAg/g could be consumed to deliver the 1 mg dose, levels which have already been achieved in small-scale production.

11.4.8 Oral Tolerance

Much debate has surrounded the incidence of tolerance as a result of vaccination, and there is evidence that tolerance develops under specific conditions, whether induced by parenteral or oral vaccination. When administering parenteral vaccines, low-zone and high-zone tolerance can be induced when a too low, or too high, booster dose follows a given primary dose (Murphy et al. 2008). When

administering oral antigens, at least two different conditions seem to induce tolerance. Early studies showed that a single high dose (between 2 and 25 mg) or several lower doses (1 mg) could induce oral tolerance to ovalbumin or hen egg lysozyme (Friedman and Weiner 1994; Mowat et al. 1982). Subsequently, frequent feedings as a method of inducing tolerance has been exploited by plant research groups seeking to reduce allergic responses to common allergens such as house dust mite allergens Der p 1 and Der f 2 (Suzuki et al. 2011; Yang et al. 2012). In these studies, mice were fed transgenic rice expressing a given allergen for seven consecutive days at a dose of approximately 1–5 mg per day. Upon subsequent injection with the allergen, IgG and IgE responses were not significantly different than non-fed, non-injected mice and were 3–4 times lower than mice fed non-transgenic rice and injected with the allergen. Tolerance has also been induced against Japanese cedar pollen by expressing major T cell epitopes for Cry j I and Cry j II. Fusion proteins were expressed in rice grains and fed to mice in either 70 μg or 560 μg doses daily for 1 month to induce tolerance (Takagi et al. 2005a, b).

These dynamics have not been explored in detail for oral vaccines, but it is clear that when an oral antigen is administered too many days in a row, the efficacy of the vaccine is lost. For example, piglets fed a 2 mg dose of maize-produced TGEV spike protein for four consecutive days showed full protection against pathogenic challenge, whereas piglets fed the same material 16 consecutive days showed significantly less protection, indicative of oral tolerance development (Lamphear et al. 2002).

One method for potentiating oral tolerance is the fusion of the antigen to the cholera toxin B subunit (CTB), coupled with less frequent oral doses over longer periods of time. Traditionally, CTB has been characterized as an adjuvant to enhance mucosal vaccine immunogenicity (Holmgren et al. 1994), but there is a mounting body of evidence that CTB can also act to augment the tolerance response to antigens (Odumosu et al. 2011; Sun et al. 2010). For example, tolerance to human coagulation factor IX (FIX) was induced in mice by feeding chloroplast-derived CTB-FIX fusion products twice a week for 8 weeks in approximately 0.13–2.0 μg doses (Verma et al. 2010). Similarly, CTB-proinsulin fusion proteins fed in 14–30 μg doses once a week for either 4 or 7 weeks induced tolerance in mice (Arakawa et al. 1998; Ruhlman et al. 2007). This approach has proven efficacious in human clinical trials for patients with Behcet's disease, a disease in which the body mounts an immune response to peptide 336–351 of human heat shock protein 60. When orally fed 0.5 mg or 5 mg of the peptide-CTB, three times per week for 12 weeks, 5 out of 8 patients remained free of disease symptoms for the length of the study (Stanford et al. 2004).

These studies illustrate that oral tolerance can be induced with various feeding regimens that are likely dependent on the dosing frequency, the amount of antigen per dose, and whether an adjuvant is used. To avoid induction of oral tolerance in mucosal vaccines, development of oral vaccine candidates will require careful vetting of adjuvants and their immunological responses under defined dosing regimens. Dosing requirements are likely antigen-specific therefore further studies will be needed to define conditions under which an orally administered HBsAg could induce tolerance.

11.5 Hurdles to Commercialization

11.5.1 Regulatory

Human clinical testing and eventual worldwide commercialization for either a primary or booster oral vaccine would involve multiple regulating bodies on several continents. In the interest of providing an initial framework for worldwide commercialization, this review will focus on the regulations set out by the FDA in the USA for Phase 1, 2, and 3 clinical trials. In addition, oversight afforded by the US Department of Agriculture (USDA) will be examined since compliance under this second regulatory agency is a prerequisite for all plant biotechnology products.

11.5.1.1 USDA

From the inception of research involving plant-produced recombinant proteins, the USDA is integral to ensuring that safe practices are followed for the growth, harvest, and storage of plants. Of primary concern is the inadvertent introduction of recombinant material into food or feed sources. In the case of other food organisms, such as eggs and yeast, vaccines have been successfully isolated from food and feed streams of production, and therefore introducing plant-based vaccines into the production chain is logistically feasible. Other plant-based, non-food proteins have been marketed (see other book chapters), demonstrating that plants can safely be used as production platforms.

One of the biggest differences between plants and other food organisms is the ability to cross-pollinate. This feature has added a host of additional requirements specific to plants. The USDA has established additional regulatory guidelines for all plant-based pharmaceuticals including vaccines. This involves a safety assessment of the recombinant product and the processing steps, as well as an environmental risk assessment before permits for the work are issued. To ensure containment, all work with the regulated plant material must follow predetermined operating procedures and must be documented at all stages of propagation, harvest, and storage. Sufficient isolation from other crops is required, as is a strict accounting of the acreage planted, the genetic identity of recombinant plants, the amount of material collected, stored, and retrieved for research or processing, and chain-of-custody documentation for movement of any plant material to other locations. For maize, the USDA requires a 1 mile (1.6 km) isolation zone from all other maize crops, an abundantly cautious requirement considering pollen has a high settling rate and viable pollen has not been detected more than 200 m from source plants (Luna et al. 2001) and is relatively short-lived. Isolation is also ensured by imposing a 50 foot buffer zone between the planted maize and other surrounding crops. Isolation of recombinant products can be further enhanced by producing the protein in a tissue-specific manner, such that recombinant protein is not detectable in nontarget tissues.

In addition to isolation, containment of the seed within regulated areas is ensured by transporting transgenic material in closed, labeled containers inside fully enclosed vehicles when site-to-site transport is required. Further containment can be achieved by devitalizing the seed onsite by grinding or other processes and transporting the nonviable processed seed to a site of choice. All seed is stored in restricted access areas where only select authorized personnel can retrieve seed.

The USDA also controls the oversight of all regulatory applications, approval of planting and transportation permits, review of standard operating procedures (SOPs), and inspection of sites for compliance. Applications undergo a rigorous review process that can last 4 or more months before a permit can be issued. Separate permits must be granted for each growth cycle, and inspections of USDA-approved sites typically occur several times a year during planting, pollination, harvest, and fallow periods.

The USDA's high level of scrutiny ensures that an exceptional level of oversight is observed for these crops. This obviously requires some administrative infrastructure for SOP documentation, authoring of yearly permits, and record-keeping for field activities, but these stipulations are not significant barriers to product development or commercialization.

11.5.1.2 FDA

A more significant hurdle to commercialization is the cost of preclinical and clinical trials. In order for the FDA to allow Phase 1 clinical trials to proceed, extensive preclinical studies are required in an animal model system that establishes the safety of the candidate. Safety is established by an absence of adverse events and toxicity to organ systems when the vaccine candidate is delivered at doses equivalent to, or exceeding, the proposed dose in human volunteers. Ideally the highest doses are tenfold greater than required in humans, but these are difficult to achieve, given the large dose of antigen needed to elicit a robust response in mice. Many mouse studies have been conducted to date (see Table 11.1), but preclinical studies used for Phase 1 applications need to show quantifiably reliable results, supported by validated assays. Validation of assays requires establishment of GLP standards which adds a layer of cost to animal trials that already require significant levels of investment.

Once preclinical trials are completed, an extensive document, the Investigational New Drug (IND) application, is submitted to the FDA for review. This document includes data for all preclinical trials and supporting protocols used to make and administer the product. In addition, the IND contains a protocol for the proposed Phase 1 human clinical trials as well as sample consent forms and an investigator's brochure outlining the drug description, formulation, and safety profile.

Safety is of primary concern to the FDA therefore the IND must discuss in detail potential safety issues related to the use of the candidate vaccine product. The HBsAg parenteral vaccine has a superior safety profile and is used routinely to immunize neonates therefore the antigen should be considered a low-risk drug, which should help build a strong case for conducting Phase 1 trials.

The preparation of the IND requires significant time and energy and rallies the expertise of project leaders, laboratory scientists, statisticians, and physicians projected to conduct Phase 1 trials. It is not uncommon to collect preclinical data 2 or more years before a projected human clinical trial and to spend 6 or more months writing and assembling the IND application. Before the IND is submitted, a pre-IND meeting is organized in which the FDA meets with the applicant and answers questions in writing posed by the applicant. The FDA requires 1–2 months to review the application and provide comments before a pre-IND meeting. Once the IND is submitted, the FDA has 30 days to review the entire submission and raise safety concerns. If no clinical holds are placed on the application, the product is allowed to enter Phase 1 clinical trials.

Phase 1 trials are designed to assess whether the drug has frequent side effects in a relatively small number of subjects, typically 20–80 (http://www.fda.gov/drugs/resourcesforyou/consumers/ucm143534.htm). In Phase 2 trials, no more than 300 individuals are recruited to test the effectiveness and safety of the drug compared to other drugs and/or a placebo. These studies would most likely compare the parenteral HBsAg vaccine to the oral vaccine, but are unlikely to include a placebo since an effective commercialized product is available for comparison. Alternatively, volunteers treated with a placebo could be offered vaccination with the commercial vaccine at the conclusion of the study. The effectiveness of an oral vaccine as a booster should be fairly straightforward to test in US populations since most young adults have received the injected vaccine at birth. Nonresponding or poor-responding populations could also be recruited to test the efficacy of the booster relative to the injected booster.

Phase 3 trials are an extension of the Phase 2 trials in terms of safety and efficacy, with up to 3,000 volunteers enrolled in the study. These studies are designed to detect more rare adverse effects of a drug and the effectiveness in a larger population. All of the considerations for Phase 2 also apply to Phase 3 trials with a particular focus on safety. The FDA recently reviewed a promising new formulation for the parenteral hepatitis B vaccine, HEPLISAV. When compared to Engerix-B®, the new formulation showed outstanding efficacy, increasing the amplitude of the immune response, decreasing the number of injections required, decreasing the time required to induce a response, and increasing seroconversion rates in poor-responding populations (Heyward et al. 2013; Janssen et al. 2013; Sablan et al. 2012). The FDA has not approved this adjuvant for use in the general population following Phase 3 clinical trials, citing a need for additional safety data to rule out the possibility of autoimmune disease causality. Of the 2,449 participants, 1,968 were treated with HEPLISAV and three developed mild-to-moderate new-onset autoimmune events, while none of the 481 participants treated with Engerix-B® developed autoimmune events. The FDA's caution in light of these data highlights the level of safety required by the FDA before allowing commercialization of a product.

Given the FDA's justifiably conservative approach to safety and the cost associated with Phase 1, 2, and 3 clinical trials, companies are somewhat recalcitrant to adopting new models of product development. This may be one of the more

important hurdles to commercialization, but not insurmountable. Several pharmaceuticals have already been produced in maize and marketed successfully, and maize-produced vaccines are efficacious in humans and livestock (Lamphear et al. 2002, 2004; Loza-Rubio et al. 2012; Tacket et al. 2004). It is predicted that once the first plant-made human vaccine is demonstrated to be safe and efficacious, subsequent plant vaccines will more easily find willing investors in the technology.

The regulatory path to commercializing a plant-based HBsAg boosting dose seems straightforward. Plant-produced HBsAg as a primary dose, however, may require a more careful design of human clinical trials to ensure that those receiving a primary dose are naive to HBsAg challenge (either by natural infection or by vaccination) and to avoid ethical conundrums. When recruiting individuals to these types of trials, it is more difficult to establish an absence of hepatitis B vaccination or exposure to HBV than it is to establish previous vaccination. Undetectable anti-HBsAg titers and consulting vaccination records may provide a first filter to recruiting volunteers for testing oral primary doses but cannot exclude previous exposure to the antigen, especially if infection occurred in the distant past. Another complication to testing mucosal primary vaccines is the ethical dilemma faced when testing an unproven candidate versus administration of a highly efficacious commercial vaccine. It may be possible to identify segments of the population for whom a parenteral vaccine is not an option, because of religious beliefs (CDC 2000; Isaacs et al. 2011), known anaphylaxis to yeast components in commercialized vaccines (DiMiceli et al. 2006), or where problems of vaccine compliance arise in remote locations, poor countries, or underserved populations (Lu et al. 2011; Lum et al. 2003; MacKellar et al. 2001; Nyamathi et al. 2012; Ouédraogo et al. 2013).

11.5.2 Production Model

Biopharmaceuticals have historically relied on microbial fermentation, mammalian cell culture, or chicken egg production models. Adding another production paradigm has typically required a significant investment of energy on the part of its champions. Manufacturing vaccines in plants is quite different from microbial and animal systems as it requires expertise in plant breeding and plant pest management. Fortunately there is a century-long tradition of agricultural farming around the world, and growers can be called upon for their extensive experience in plant production. In addition, large-scale production scenarios for maize can be implemented with relatively low start-up capital investment costs when compared to the establishment of fermentation facilities used for microbial production (Howard et al. 2011). Although these cost models are applied to industrial enzyme production, the magnitude in capital investment savings can be equally applied to biopharmaceutical production.

One advantage of using a plant-based production system over mammalian cell or chicken egg production systems is the reduced risk of infection by potential human pathogens. Contamination has plagued the production of biopharmaceuticals made

in animal cells and has led to serious detrimental effects in vaccinated animals. It has led to wasting syndrome and diarrhea in pigs, scrapie in sheep, and abortion and death in canines (Pastoret 2010). Contamination has also led to product shortages of human therapeutics. Most recently, Genzyme discovered Vesivirus 2117 in its production line in 2008 and 2009, resulting in an alarming worldwide shortage of pharmaceutical therapeutics for rare diseases (Allison 2010). These situations would not arise in a plant production system since plant cells do not harbor animal viruses or prions.

11.5.3 Education

Public perception plays a key role in commercialization, and efforts should be made to engage and educate the public about plant-based systems. Adequate isolation of biopharmaceutical crops from food crops, assurance of containment, and land use for biopharmaceutical production versus food production are all issues of concern to the public and with proper consideration can strengthen a case for biopharmaceuticals in plants.

The use of organisms that are traditionally used in food for the production of biopharmaceuticals is not new to the vaccine industry. Yeast and eggs are sources of both vaccines and food products and maintain separate production systems without cross contamination, a scenario which is equally applicable to plant systems. As discussed above (see Sect. 15.5.1.), all transgenic plants in the USA are regulated by the USDA, a government agency that strictly enforces containment policies to ensure that biopharmaceutical crops remain separate from plants destined for food or feed. In addition, plant-made pharmaceuticals must also be vetted by the FDA, to ensure efficacy and safety of the product before it can be licensed and commercialized. Ultimately the public's perception of the USDA and FDA as competent regulatory entities will go a long way toward promoting acceptance of plant-based biopharmaceuticals within the general public.

Currently >35 % of all corn is cultivated for the production of biofuels. With the shift in maize production from food to biofuels and the associated increase in food prices, the public is understandably wary of new uses for crop plants that may further increase food prices and impose shortages in food-producing agricultural land. Using maize as a system for vaccine production, however, represents a very small fraction of the total land used for corn production. With a maize crop expressing 100 mg HBsAg/kg grain, producing 4,000 kg of grain per acre, and a requirement of 1 mg HBsAg per dose, one acre could produce 400,000 doses. To distribute a vaccine dose to the entire US population (300 million individuals) would require 750 acres, which represents 0.0009 % of the total acreage in the USA (80 million acres of maize in production). Therefore, even with 100 different vaccines in production, the use of maize as a production system could provide ten doses to each US citizen every year using less than 1 % of the total maize acreage. This obviously provides an overestimate of the demand for an HBsAg vaccine since

most individuals require three doses every 20 years, but it does illustrate the potential for the production of other vaccines and for providing a vaccine supply to other parts of the world without overtaxing the US agricultural land base.

11.6 Advantages of a Maize Seed Production System

Maize germ has emerged as the front-runner candidate for an oral hepatitis B vaccine on several fronts. As mentioned above, it produces the highest concentrations of HBsAg antigen of all the plant oral delivery systems, and it induces a robust immunologic response in mice without the need for an adjuvant. The HBsAg antigen can be produced in a cost-effective manner, has a superior stability profile in germ, and seems to adopt a structural conformation that is highly immunogenic. Dried maize germ eliminates the need for a cold chain and can be conveniently administered by minimally trained health professionals in a convenient formulation.

Maize germ also has a proven track record for delivering immunogenic and protective oral vaccines. The transmissible gastroenteritis virus (TGEV) spike protein expressed in germ and fed to pigs conferred protection against TGEV as well as, if not better than, the injected commercial vaccine (Lamphear et al. 2002, 2004). Piglets fed transgenic maize for four days and challenged with TGEV showed no signs of disease, while piglets administered the parenteral vaccine showed a 10 % incidence of morbidity and piglets fed control maize showed a 50 % incidence of morbidity. Protection has also been seen in ruminants, animals with multiple stomach compartments that likely expose the antigen to additional degradation pressures. Maize kernels expressing the rabies G-protein were able to protect lambs against lethal challenge with a single oral dose (2 mg of antigen in 80 g of maize material), and survival rates following oral vaccination were equivalent to rates in animals receiving the commercial parenteral vaccine (Loza-Rubio et al. 2012). Maize was also successfully used to express the heat labile enterotoxin (LT) of enterotoxigenic *E. coli*. When administered orally to human volunteers, the vaccine proved immunogenic, inducing increased anti-LT serum IgA and IgG, and mucosal IgA, as detected in the subjects' stool (Tacket et al. 2004). This human clinical trial also demonstrated safety of the orally administered maize material in human volunteers, as no severe adverse events were reported.

Scale-up of maize production is surprisingly quick, providing plentiful material that can be stored for long periods of time in a relatively short timeframe. Assuming a seed set of 400 seeds per year and a 3-month long growing season, a 60-million fold scale-up of maize material can be achieved in 1 year. Large scale-up with each generation confers an added advantage over microbial systems and over plant systems with smaller seed sets: reduced risk of random mutations. For production of proteins in microbial systems, cells divide approximately every 20 min to produce large quantities of recombinant protein, offering ample opportunity for random mutations to arise, potentially compromising the antigen nucleotide

sequence and protein product. In a maize system, gametes are produced during a very short time in development and undergo a relatively small number of divisions compared to their microbial counterparts. In essence this reduces the risk of de novo mutations in the gamete population and therefore greatly reduces the risk of mutations passed on to subsequent generations.

The absence of maize wild relatives in the USA is also an attractive feature of this system. Without the potential for outcrossing, introduction of the transgene into the surrounding ecosystem is essentially mitigated. This is not the case for all countries, most notably Mexico, where the wild relative of maize, teosinte, is distributed from the midline of the northern state of Chihuahua, throughout central Mexico, and down to the Guatemalan border (Sánchez González and Ruiz Corral 1997).

From a safety standpoint, maize confers several advantages over other edible plants. Unlike some cereal grains, it does not contain problematic proteins, such as gluten, that can be life-threatening for celiac disease (CD) patients. Individuals with CD are a group of poor responders to the parenteral vaccine, displaying seroconversion in 50–68 % of vaccines (Ahishali et al. 2008; Leonardi et al. 2009). One in 133 people show signs of CD (Fasano et al. 2003) therefore a gluten-free vaccine option is essential for this subpopulation. Maize grain also has a superior food safety profile and has been granted GRAS status by the FDA, a status which recognizes its widespread safe use as a food. Maize grain contains no known toxic secondary compounds or anti-nutritional compounds, and therefore the grain does not need processing or purification before consumption. In terms of allergenicity, prevalence is still unknown (Venter et al. 2008). It is clear, however, that it is not listed as a major food allergen by either the USA (FDA 2010) or the European Union (European Commission 2000). Presently, the CDC recommends that individuals with hypersensitivity to yeast forgo the hepatitis B parenteral vaccine (CDC 2006) due to the risk of adverse reactions to yeast proteins remaining in the vaccine. The same recommendations would be in place for a maize-based vaccine concerning individuals with maize allergies.

11.7 Future Directions

The prospect of using an HBsAg oral vaccine as a booster seems imminent, but use as a primary dose will require additional proof of principle studies. Early studies in mice by Kong et al. (2001) showed that oral delivery of potato-produced HBsAg as a primer followed by a yeast-derived injected booster led to very high anti-HBsAg titers (3,300 mIU/mL). It remains to be seen whether these high titers can be achieved in mice or humans when an oral vaccine is used as both the primary and booster doses and whether titers can be maintained for decades, as is the case with the parenteral vaccine.

Until recently it has been difficult to obtain comparable immunologic responses between parenteral and oral delivery systems in mice due to suboptimal HBsAg

concentrations delivered orally. With higher expressing maize material, it is now possible to explore the effect of oral delivery on mucosal and systemic responses and compare them to parenteral responses. It is quite possible that oral delivery will produce a more robust mucosal response, while parenteral delivery will produce a stronger systemic response. Since the present standard for protection, >10 mIU/mL, is based on serum antibody titers, use of an oral vaccine may require a reexamination of this standard to include biologically relevant levels for mucosal immunity, perhaps in alternate fluids, such as saliva, that reflect mucosal immunity more accurately.

Plant-based systems can also be used to produce other subunit vaccines at a low cost per dose for human or animal health. Plant-based systems can also be used to produce other vaccines at a low cost per dose for human or animal health. Vaccines need not be limited to viral disease, since invertebrate proteins have been successfully applied as vaccines against tick and nematode infestations in cattle and sheep (Piedrafita et al. 2012; Vargas et al. 2010). Oral delivery systems that are heat stable would be particularly useful in these settings since many ranchers work in remote locations where refrigeration and the need for repeat immunizations present logistical challenges (Meeusen et al. 2007).

Another largely unexplored area of research lies in the use of effective oral adjuvants or carrier proteins to boost the immunologic response. Parenteral vaccines have been paired with many non-alum adjuvants, and only now promising candidates are emerging. Of note, the 1018 immunostimulatory sequence (ISS) championed by Dynavax Technologies has improved seroprotection rates, increased titers, and decreased response times when combined with the HBsAg antigen (Halperin et al. 2012a, b; Sablan et al. 2012). Similar adjuvants that could survive the environment of the GI tract could be useful for oral vaccines. Carrier proteins may also provide added immunologic impact. These proteins or peptides are typically fused to the antigen and target the antigen to specific cells in the gut. For example, the DC peptide was used as a carrier for the *Bacillus anthracis* protective antigen (PA). A DC-PA fusion protein was expressed in *Lactobacillus acidophilus* and bacterial suspensions were fed to mice (Mohamadzadeh et al. 2009). Following pathogenic challenge, survival of the DC-PA-fed mice was greatly improved compared to PA-fed mice (75 % versus 25 %, respectively). Carrier proteins of this type could easily be incorporated into a plant expression system.

11.8 Overall Significance

A plant-produced oral vaccine has the potential to achieve many, if not all, attributes of an ideal vaccine outlined at the beginning of this chapter. The ease of transport, storage, distribution, and cost savings of a maize-based vaccine could significantly improve vaccine access to remote areas and could redistribute economic resources to other vaccine programs that are in need of funding. An oral-

based vaccine would also induce immunity at the mucosa, sites of likely infection for many individuals, especially those who acquire the disease via sexual contact. In many ways, the hepatitis B oral vaccine is a proof of concept product and could lead the way to other subunit vaccines being produced in a more affordable, convenient vehicle for delivery.

The hope then is for eradication of hepatitis B in the not-too-distant future. Humans are the only known reservoir of HBV, with no reported incidences of nonhuman primate strains of HBV infecting humans (Ghendon 1990; Sa-Nguanmoo et al. 2009). Vaccination and treatment efforts can therefore be focused on human populations without the need for control in wild animal populations. Eradication will require implementation of universal neonate immunizations, a periodic boosting regime at 20-year intervals, improved vaccine response in poor and nonresponders, and treatment of existing chronic carriers for clearance of the infection. Oral vaccines have the potential to revolutionize the administration of boosting treatments, especially in remote areas, and may deliver a more immunogenic product to traditional nonresponders.

References

Ahishali E, Boztas G, Akyuz F, Ibrisim D, Poturoglu S, Pinarbasi B, Ozdil S, Mungan Z (2008) Response to hepatitis B vaccination in patients with Celiac disease. Dig Dis Sci 53(8):2156–2159

Allison M (2010) As Genzyme flounders, competitors and activist investors swoop in. Nat Biotechnol 28(1):3–4

Arakawa T, Timasheff SN (1982) Stabilization of protein structure by sugars. Biochemistry 21(25):6536–6544

Arakawa T, Yu J, Chong DK, Hough J, Engen PC, Langridge WH (1998) A plant-based cholera toxin B subunit–insulin fusion protein protects against the development of autoimmune diabetes. Nat Biotechnol 16(10):934–938

Bailey MR (2000) A model system for edible vaccination using recombinant avidin produced in corn seed. Texas A&M, College Station, TX

Behre U, Bleckmann G, Crasta PD, Leyssen M, Messier M, Jacquet JM, Hardt K (2012) Long-term anti-HBs antibody persistence and immune memory in children and adolescents who received routine childhood hepatitis B vaccination. Hum Vaccin Immunother 8(6):704–705

BioTechnology General (2001) BIO-HEP-B prescribing information. http://www.novaccine.com/pdffiles/BIO-HEP-B-package_insert.pdf. Accessed February 13, 2013

Boisen S (1983) Protease inhibitors in cereals. Acta Agriculturae Scandinavica 33(4):369–381

Bond WW, Petersen NJ, Favero MS (1977) Viral hepatitis B: aspects of environmental control. Health Lab Sci 14(4):235–252

Borges O, Tavares J, de Sousa A, Borchard G, Junginger HE, Cordeiro-da-Silva A (2007) Evaluation of the immune response following a short oral vaccination schedule with hepatitis B antigen encapsulated into alginate-coated chitosan nanoparticles. Eur J Pharm Sci 32(4):278–290

Carman WF, Karayiannis P, Waters J, Thomas H, Zanetti A, Manzillo G, At Z (1990) Vaccine-induced escape mutant of hepatitis B virus. The Lancet 336(8711):325–329

CDC (2000) Measles outbreak – Netherlands, April 1999–January 2000. MMWR 49(14):299–303

CDC (2006) A comprehensive immunization strategy to eliminate transmission of hepatitis B virus infection in the United States. Pediatrics 118(1):404

CDC (2011) Use of hepatitis B vaccination for adults with diabetes mellitus: recommendations of the Advisory Committee on Immunization Practices (ACIP). MMWR 60(50):1709–1711
CDC (2013) CDC—VFC current vaccine price list. http://www.cdc.gov/vaccines/programs/vfc/awardees/vaccine-management/price-list/index.html. Accessed February 2013
Chaves SS, Daniels D, Cooper BW, Malo-Schlegel S, MacArthur S, Robbins KC, Kobetitsch JF, McDaniel A, D'Avella JF, Alter MJ (2011) Immunogenicity of hepatitis B vaccine among hemodialysis patients: effect of revaccination of non-responders and duration of protection. Vaccine 29(52):9618–9623
Chaves SS, Fischer G, Groeger J, Patel PR, Thompson ND, Teshale EH, Stevenson K, Yano VM, Armstrong GL, Samandari T (2012) Persistence of long-term immunity to hepatitis B among adolescents immunized at birth. Vaccine 30(9):1644–1649
Czerkinsky C, Holmgren J (2012) Mucosal delivery routes for optimal immunization: targeting immunity to the right tissues. In: Kozlowski P (ed) Mucosal vaccines: modern concepts, strategies, and challenges, vol 354, Current topics in microbiology and immunology. Springer, Heidelberg, pp 1–18
Dannetun E, Tegnell A, Torner A, Giesecke J (2006) Coverage of hepatitis B vaccination in Swedish healthcare workers. J Hosp Infect 63(2):201–204
DiMiceli L, Pool V, Kelso JM, Shadomy SV, Iskander J, Team V (2006) Vaccination of yeast sensitive individuals: review of safety data in the US vaccine adverse event reporting system (VAERS). Vaccine 24(6):703–707
Durrheim DN, Poland GA (2013) United Nations mercury treaty jeopardizes vaccine protection of the world's most vulnerable children. Vaccine 31(10):1357–1358
Edmunds WJ, Medley GF, Nokes DJ, Hall AJ, Whittle HC (1993) The influence of age on the development of hepatitis b carrier state. Proc R Soc B-Biol Sci 253(1337):197–201
European Commission (2000) Directive 2000/13/EC. http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CONSLEG:2000L0013:20110120:EN:PDF
Evangelista RL, Kusnadi AR, Howard JA, Nikolov ZL (2008) Process and economic evaluation of the extraction and purification of recombinant β-glucuronidase from transgenic corn. Biotechnol Prog 14(4):607–614
Fasano A, Berti I, Gerarduzzi T, Not T, Colletti RB, Drago S, Elitsur Y, Green PH, Guandalini S, Hill ID (2003) Prevalence of celiac disease in at-risk and not-at-risk groups in the United States: a large multicenter study. Arch Intern Med 163(3):286–292
FDA (2010) Food allergies: what you need to know. http://www.fda.gov/downloads/Food/ResourcesForYou/Consumers/UCM220117.pdf. Accessed February 2013
Fischer R, Stoger E, Schillberg S, Christou P, Twyman RM (2004) Plant-based production of biopharmaceuticals. Curr Opin Plant Biol 7(2):152–158
Friedman A, Weiner HL (1994) Induction of anergy or active suppression following oral tolerance is determined by antigen dosage. Proc Natl Acad Sci U S A 91(14):6688–6692
Gao Y, Ma Y, Li M, Cheng T, Li S-W, Zhang J, Xia N-S (2003) Oral immunization of animals with transgenic cherry tomatillo expressing HBsAg. World J Gastroenterol 9(5):996–1002
GAVI Alliance (2012) GAVI facts and figures, November 2012. www.gavialliance.org/library/publications/gavi-fact-sheets/gavi-facts-and-figures. Accessed February 2013
Ghendon Y (1990) WHO strategy for the global elimination of new cases of hepatitis B. Vaccine 8: S129–S133
Grisshammer R (2006) Understanding recombinant expression of membrane proteins. Curr Opin Biotechnol 17(4):337–340
GSK (1989) Engerix-B prescribing information. http://us.gsk.com/products/assets/us_engerixb.pdf.
GSK (2001) Twinrix prescribing information. http://us.gsk.com/products/assets/us_twinrix.pdf. Accessed February 13, 2013
Gupta PN, Khatri K, Goyal AK, Mishra N, Vyas SP (2007) M-cell targeted biodegradable PLGA nanoparticles for oral immunization against hepatitis B. J Drug Target 15(10):701–713

Hall AJ, Roberston RL, Crivelli PE, Lowe Y, Inskip H, Snow SK, Whittle H (1993) Cost-effectiveness of hepatitis B vaccine in The Gambia. Trans R Soc Trop Med Hyg 87(3):333–336

Halperin SA, McNeil S, Langley JM, Smith B, MacKinnon-Cameron D, McCall-Sani R, Heyward WL, Martin JT (2012a) Safety and immunogenicity of different two-dose regimens of an investigational hepatitis B vaccine (hepatitis B surface antigen co-administered with an immunostimulatory phosphorothioate oligodeoxyribonucleotide) in healthy young adults. Vaccine 30(36):5445–5448

Halperin SA, Ward B, Cooper C, Predy G, Diaz-Mitoma F, Dionne M, Embree J, McGeer A, Zickler P, Moltz K-H (2012b) Comparison of safety and immunogenicity of two doses of investigational hepatitis B virus surface antigen co-administered with an immunostimulatory phosphorothioate oligodeoxyribonucleotide and three doses of a licensed hepatitis B vaccine in healthy adults 18–55 years of age. Vaccine 30(15):2556–2563

Hayden CA, Egelkrout EM, Moscoso AM, Enrique C, Keener TK, Jimenez-Flores R, Wong JC, Howard JA (2012a) Production of highly concentrated, heat-stable hepatitis B surface antigen in maize. Plant Biotechnol J 10(8):979–984. doi:10.1111/j.1467-7652.2012.00727.x

Hayden CA, Streatfield SJ, Lamphear BJ, Fake GM, Keener TK, Walker JH, Clements JD, Turner DD, Tizard IR, Howard JA (2012b) Bioencapsulation of the hepatitis B surface antigen and its use as an effective oral immunogen. Vaccine 30(19):2937–2942

He X-W, Wang F, Jiang L, Li J, S-k L, Xiao Z-Y, Jin X-Q, Zhang Y-N, He Y, Li K (2005) Induction of mucosal and systemic immune response by single-dose oral immunization with biodegradable microparticles containing DNA encoding HBsAg. J Gen Virol 86(3):601–610

Heyward WL, Kyle M, Blumenau J, Davis M, Reisinger K, Kabongo ML, Bennett S, Janssen RS, Namini H, Martin JT (2013) Immunogenicity and safety of an investigational hepatitis B vaccine with a Toll-like receptor 9 agonist adjuvant (HBsAg-1018) compared to a licensed hepatitis B vaccine in healthy adults 40–70 years of age. Vaccine 31:5300–5305. doi:10.1016/j.vaccine.2013.05.068

Hipgrave DB, Tran TN, Huong VM, Dat DT, Nga NT, Long HT, Van NT, Maynard JE, Biggs B-A (2006) Immunogenicity of a locally produced hepatitis B vaccine with the birth dose stored outside the cold chain in rural Vietnam. Am J Trop Med Hyg 74(2):255–260

Holmgren J, Czerkinsky C, Lycke N, Svennerholm A-M (1994) Strategies for the induction of immune responses at mucosal surfaces making use of cholera toxin B subunit as immunogen, carrier, and adjuvant. Am J Trop Med Hyg 50(5 Suppl):42–54

Hood EE, Woodard SL, Horn ME (2002) Monoclonal antibody manufacturing in transgenic plants—myths and realities. Curr Opin Biotechnol 13(6):630–635

Howard JA, Hood EE (2007) Methods for growing nonfood products in transgenic plants. Crop Sci 47(3):1255–1262

Howard JA, Nikolov Z, Hood EE (2011) Enzyme production systems for biomass conversion. In: Hood EE, Nelson P, Powell R (eds) Plant biomass conversion. Wiley, Somerset, NJ, pp 227–253

Huang Z, LePore K, Elkin G, Thanavala Y, Mason HS (2008) High-yield rapid production of hepatitis B surface antigen in plant leaf by a viral expression system. Plant Biotechnol J 6(2):202–209

Hur K, Wong M, Lee J, Lee J, Juon H-S (2012) Hepatitis B infection in the Asian and Latino communities of Alameda County, California. J Community Health 37:1–8

Hyams KC (1995) Risks of chronicity following acute hepatitis B virus infection: a review. Clin Infect Dis 20(4):992–1000

Isaacs D, Kilham HA, Alexander S, Wood N, Buckmaster A, Royle J (2011) Ethical issues in preventing mother-to-child transmission of hepatitis B by immunisation. Vaccine 29(37):6159–6162

Jain AK, Goyal AK, Mishra N, Vaidya B, Mangal S, Vyas SP (2010) PEG–PLA–PEG block copolymeric nanoparticles for oral immunization against hepatitis B. Int J Pharm 387(1):253–262

Janssen RS, Mangoo-Karim R, Pergola PE, Girndt M, Namini H, Rahman S, Bennett SR, Heyward WL, Martin JT (2013) Immunogenicity and safety of an investigational hepatitis B vaccine with a toll-like receptor 9 agonist adjuvant (HBsAg-1018) compared with a licensed hepatitis B vaccine in patients with chronic kidney disease. Vaccine 31:5306–5313

Joung Y, Youm J, Jeon J, Lee B, Ryu C, Hong H, Kim H, Joung H, Kim H (2004) Expression of the hepatitis B surface S and preS2 antigens in tubers of Solanum tuberosum. Plant Cell Rep 22(12):925–930

Kamara L, Lydon P, Bilous J, Vandelaer J, Eggers R, Gacic-Dobo M, Meaney W, Okwo-Bele J-M (2012) Global immunization vision and strategy (GIVS): a mid-term analysis of progress in 50 countries. Health Policy Plan 28:1–9

Kamili S, Sozzi V, Thompson G, Campbell K, Walker CM, Locarnini S, Krawczynski K (2008) Efficacy of hepatitis B vaccine against antiviral drug-resistant hepatitis B virus mutants in the chimpanzee model. Hepatology 49(5):1483–1491

Kapusta J, Modelska A, Figlerowicz M, Pniewski T, Letellier M, Lisowa O, Yusibov V, Koprowski H, Plucienniczak A, Legocki A (1999) A plant-derived edible vaccine against hepatitis B virus. FASEB J 13(13):1796–1799

Keating GM, Noble S (2003) Recombinant hepatitis B vaccine (Engerix-B (R))—a review of its immunogenicity and protective efficacy against hepatitis B. Drugs 63(10):1021–1051

Kong Q, Richter L, Yang YF, Arntzen CJ, Mason HS, Thanavala Y (2001) Oral immunization with hepatitis B surface antigen expressed in transgenic plants. Proc Natl Acad Sci U S A 98 (20):11539–11544

Lamphear BJ, Streatfield SJ, Jilka JM, Brooks CA, Barker DK, Turner DD, Delaney DE, Garcia M, Wiggins B, Woodard SL, Hood EE, Tizard IR, Lawhorn B, Howard JA (2002) Delivery of subunit vaccines in maize seed. J Control Release 85(1–3):169–180

Lamphear BJ, Jilka JM, Kesl L, Welter M, Howard JA, Streatfield SJ (2004) A corn-based delivery system for animal vaccines: an oral transmissible gastroenteritis virus vaccine boosts lacto-genic immunity in swine. Vaccine 22(19):2420–2424

Landrum ML, Hullsiek KH, O'Connell RJ, Chun HM, Ganesan A, Okulicz JF, Lalani T, Weintrob AC, Crum-Cianflone NF, Agan BK (2012) Hepatitis B vaccine antibody response and the risk of clinical AIDS or death. PLoS One 7(3):e33488

Laurence JC (2005) Hepatitis A and B immunizations of individuals infected with human immunodeficiency virus. Am J Med 118(10):75–83

Leonardi S, Spina M, Spicuzza L, Rotolo N, La Rosa M (2009) Hepatitis B vaccination failure in celiac disease: is there a need to reassess current immunization strategies? Vaccine 27(43):6030–6033

Leonardi S, Praticò AD, Lionetti E, Spina M, Vitaliti G, La Rosa M (2012) Intramuscular vs intradermal route for hepatitis B booster vaccine in celiac children. World J Gastroenterol 18(40):5729–5733

Lepetic A, Biscayart C, Seigelchifer M, Arduino R, Stamboulian D (2003) Persistence of immunity and seroprotection 4 years after a primary vaccination schedule with a *Hansenula polymorpha* recombinant hepatitis B vaccine. Vaccine 21(27):4481–4485

Levitz RE, Cooper BW, Regan HC (1995) Immunization with high-dose intradermal recombinant hepatitis B vaccine in healthcare workers who failed to respond to intramuscular vaccination. Infect Control Hosp Epidemiol 16(2):88–91

Lin SY, Chang ET, So SK (2009) Stopping a silent killer in the underserved Asian and Pacific Islander community: a chronic hepatitis B and liver cancer prevention clinic by medical students. Asian Pac J Cancer Prev 10(3):383–386

Lou X-M, Yao Q-H, Zhang Z, Peng R-H, Xiong A-S, Wang H-K (2007) Expression of the human hepatitis B virus large surface antigen gene in transgenic tomato plants. Clin Vaccine Immunol 14(4):464–469

Loza-Rubio E, Rojas-Anaya E, López J, Olivera-Flores MT, Gómez-Lim M, Pérez GT (2012) Induction of a protective immune response to rabies virus in sheep after oral immunization with transgenic maize. Vaccine 30(27):5551–5556

Lu P-J, Byrd KK, Murphy TV, Weinbaum C (2011) Hepatitis B vaccination coverage among high-risk adults 18–49 years, US, 2009. Vaccine 29(40):7049–7057

Lubeck MD, Davis AR, Chengalvala M, Natuk RJ, Morin JE, Molnar-Kimber K, Mason BB, Bhat BM, Mizutani S, Hung PP (1989) Immunogenicity and efficacy testing in chimpanzees of an

oral hepatitis B vaccine based on live recombinant adenovirus. Proc Natl Acad Sci U S A 86(17):6763–6767
Lum PJ, Ochoa KC, Hahn JA, Shafer KP, Evans JL, Moss AR (2003) Hepatitis B virus immunization among young injection drug users in San Francisco, CA: the UFO Study. Am J Public Health 93(6):919–923
Luna V, Figueroa M, Baltazar M, Gomez L, Townsend R, Schoper J (2001) Maize pollen longevity and distance isolation requirements for effective pollen control. Crop Sci 41(5):1551–1557
MacKellar DA, Valleroy LA, Secura GM, McFarland W, Shehan D, Ford W, LaLota M, Celentano DD, Koblin BA, Torian LV (2001) Two decades after vaccine license: hepatitis B immunization and infection among young men who have sex with men. Am J Public Health 91(6):965–971
Macpherson A, McCoy K, Johansen F, Brandtzaeg P (2008) The immune geography of IgA induction and function. Mucosal Immunol 1(1):11–22
Madaliński K, Sylvan S, Hellstrom U, Mikołajewicz J, Dzierzanowska-Fangrat K (2004) Presence of anti-preS1, anti-preS2, and anti-HBs antibodies in newborns immunized with Bio-Hep-B vaccine. Med Sci Monit 10(1):PI10–PI17
Mangold CM, Unckell F, Werr M, Streeck RE (1995) Secretion and antigenicity of hepatitis B virus small envelope proteins lacking cysteines in the major antigenic region. Virology 211(2):535–543
Mangold C, Unckell F, Werr M, Streeck R (1997) Analysis of intermolecular disulfide bonds and free sulfhydryl groups in hepatitis B surface antigen particles. Arch Virol 142(11):2257–2267
Martinez-Puig D, Pérez JF, Castillo M, Andaluz A, Anguita M, Morales J, Gasa J (2003) Consumption of raw potato starch increases colon length and fecal excretion of purine bases in growing pigs. J Nutr 133(1):134–139
Mason H, Lam D, Arntzen C (1992) Expression of hepatitis B surface antigen in transgenic plants. Proc Natl Acad Sci U S A 89(24):11745–11749
McMahon BJ, Alward WLM, Hall DB, Heyward WL, Bender TR, Francis DP, Maynard JE (1985) Acute hepatitis B virus infection: relation of age to the clinical expression of disease and subsequent development of the carrier state. J Infect Dis 151(4):599–603
Meeusen EN, Walker J, Peters A, Pastoret P-P, Jungersen G (2007) Current status of veterinary vaccines. Clin Microbiol Rev 20(3):489–510
Merck (1998) Recombivax prescribing information. http://www.merck.com/product/usa/pi_circulars/r/recombivax_hb/recombivax_pi.pdf. Accessed February 13, 2013
Milich DR, McNamara MK, McLachlan A, Thornton GB, Chisari FV (1985a) Distinct H-2-linked regulation of T-cell responses to the pre-S and S regions of the same hepatitis B surface antigen polypeptide allows circumvention of nonresponsiveness to the S region. Proc Natl Acad Sci U S A 82(23):8168–8172
Milich DR, Thornton GB, Neurath AR, Kent SB, Michel M-L, Tiollais P, Chisari FV (1985b) Enhanced immunogenicity of the pre-S region of hepatitis B surface antigen. Science (New York, NY) 228(4704):1195–1199
Milich DR, McLachlan A, Chisari F, Kent S, Thorton G (1986) Immune response to the pre-S (1) region of the hepatitis B surface antigen (HBsAg): a pre-S (1)-specific T cell response can bypass nonresponsiveness to the pre-S (2) and S regions of HBsAg. J Immunol 137(1):315–322
Mohamadzadeh M, Duong T, Sandwick SJ, Hoover T, Klaenhammer TR (2009) Dendritic cell targeting of Bacillus anthracis protective antigen expressed by Lactobacillus acidophilus protects mice from lethal challenge. Proc Natl Acad Sci U S A 106(11):4331–4336. doi:10.1073/pnas.0900029106
Mowat A, Strobel S, Drummond HE, Ferguson A (1982) Immunological responses to fed protein antigens in mice. I. Reversal of oral tolerance to ovalbumin by cyclophosphamide. Immunology 45(1):105–113
Murphy K, Travers P, Walport M (2008) Janeway's immunobiology, 7th edn. Garland Science, New York, NY

Nagafuchi S, Kashiwagi S, Okada K, Anzai K, Nakamura M, Nishimura Y, Sasazuki T, Niho Y (1991) Reversal of nonresponders and postexposure prophylaxis by intradermal hepatitis B vaccination in Japanese medical personnel. JAMA 265(20):2679–2683

Nandi S, Yalda D, Lu S, Nikolov Z, Misaki R, Fujiyama K, Huang N (2005) Process development and economic evaluation of recombinant human lactoferrin expressed in rice grain. Transgenic Res 14(3):237–249

Nyamathi AM, Marlow E, Branson C, Marfisee M, Nandy K (2012) Hepatitis A/B vaccine completion among homeless adults with history of incarceration. J Forensic Nurs 8:13–22

Odumosu O, Nicholas D, Payne K, Langridge W (2011) Cholera toxin B subunit linked to glutamic acid decarboxylase suppresses dendritic cell maturation and function. Vaccine 29(46):8451–8458

Ott JJ, Stevens GA, Groeger J, Wiersma ST (2012) Global epidemiology of hepatitis B virus infection: new estimates of age-specific HBsAg seroprevalence and endemicity. Vaccine 30(12):2212–2219

Otto BF, Suarnawa I, Stewart T, Nelson C, Ruff TA, Widjaya A, Maynard JE (1999) At-birth immunisation against hepatitis B using a novel pre-filled immunisation device stored outside the cold chain. Vaccine 18(5):498–502

Ouédraogo H, Kouanda S, Tiendrébeogo S, Konseimbo G, Yetta C, Tiendrébeogo E, Savadogo A, Sondo B (2013) Hepatitis B vaccination status and associated factors among healthcare workers in Burkina Faso. Medecine et Sante Tropicales

Pastoret P-P (2010) Human and animal vaccine contaminations. Biologicals 38(3):332–334

Piedrafita D, de Veer M, Sherrard J, Kraska T, Elhay M, Meeusen E (2012) Field vaccination of sheep with a larval-specific antigen of the gastrointestinal nematode, *Haemonchus contortus*, confers significant protection against an experimental challenge infection. Vaccine 30(50):7199–7204

Pniewski T, Kapusta J, Bociąg P, Wojciechowicz J, Kostrzak A, Gdula M, Fedorowicz-Strońska O, Wójcik P, Otta H, Samardakiewicz S (2011) Low-dose oral immunization with lyophilized tissue of herbicide-resistant lettuce expressing hepatitis B surface antigen for prototype plant-derived vaccine tablet formulation. J Appl Genet 52(2):125–136

Poovorawan Y, Chongsrisawat V, Theamboonlers A, Leroux-Roels G, Crasta PD, Hardt K (2012) Persistence and immune memory to hepatitis B vaccine 20 years after primary vaccination of Thai infants, born to HBsAg and HBeAg positive mothers. Hum Vaccin Immunother 8(7):896–904

Rajkannan R, Dhanaraju M, Gopinath D, Selvaraj D, Jayakumar R (2006) Development of hepatitis B oral vaccine using B-cell epitope loaded PLG microparticles. Vaccine 24(24):5149–5157

Richter LJ, Thanavala Y, Arntzen CJ, Mason HS (2000) Production of hepatitis B surface antigen in transgenic plants for oral immunization. Nat Biotechnol 18(11):1167–1171

Roome AJ, Walsh SJ, Cartter ML, Hadler JL (1993) Hepatitis B vaccine responsiveness in Connecticut public safety personnel. JAMA 270(24):2931–2934

Roukens AH, Vossen AC, Boland GJ, Verduyn W, van Dissel JT, Visser LG (2010) Intradermal hepatitis B vaccination in non-responders after topical application of imiquimod (Aldara®). Vaccine 28(26):4288–4293

Ruhlman T, Ahangari R, Devine A, Samsam M, Daniell H (2007) Expression of cholera toxin B–proinsulin fusion protein in lettuce and tobacco chloroplasts–oral administration protects against development of insulitis in non-obese diabetic mice. Plant Biotechnol J 5(4):495–510

Rustgi VK, Schleupner CJ, Krause DS (1995) Comparative study of the immunogenicity and safety of Engerix-B administered at 0, 1, 2 and 12 months and Recombivax HB administered at 0, 1, and 6 months in healthy adults. Vaccine 13(17):1665–1668

Sablan BP, Kim DJ, Barzaga NG, Chow WC, Cho M, Ahn SH, Hwang SG, Lee JH, Namini H, Heyward WL (2012) Demonstration of safety and enhanced seroprotection against hepatitis B with investigational HBsAg-1018 ISS vaccine compared to a licensed hepatitis B vaccine. Vaccine 30:2689–2696

Salyaev R, Rekoslavskaya N, Shchelkunov S, Stolbikov A, Hammond R (2009) Study of the mucosal immune response duration in mice after administration of a candidate edible vaccine based on transgenic tomato plants carrying the TBI-HBS gene. Dokl Biochem Biophys 428(1):232–234

Sánchez González JJ, Ruiz Corral JA (1997) Teosinte distribution in Mexico. In: Serratos JA, Willcox MC, Castillo-González F (eds) Gene flow among maize landraces, improved maize varieties, and teosinte: implications for transgenic maize. CIMMYT, Mexico, DF, p 21

Sa-Nguanmoo P, Rianthavorn P, Amornsawadwattana S, Poovorawan Y (2009) Hepatitis B virus infection in non-human primates. Acta Virol 53(2):73–82

Sanofi-Pasteur (2011) HBvaxPRO prescribing information. http://www.spmsd.co.uk/file.asp?docid=195. Accessed February 13, 2013

Schillie S, Xing J, Murphy T, Hu D (2012) Prevalence of hepatitis B virus infection among persons with diagnosed diabetes mellitus in the United States, 1999–2010. J Viral Hepat 19:674–676

Scott Cassara (2009) AgB Prospecto Medico. http://www.scottcassara.com.ar/images/PDFS/AgB.pdf. Accessed February 13, 2013

Serum Institute of India (2009) Gene Vac-B prescribing information. http://www.seruminstitute.com/content/products/product_genevac.htm. Accessed February 13, 2013

Shantha (2009) Shanvac-B prescribing information. http://www.shanthabiotech.com/files/Shanvac%20B%20Domestic%20Pack%20insert.pdf. Accessed February 13, 2013

Shapira MY, Zeira E, Adler R, Shouval D (2001) Rapid seroprotection against hepatitis B following the first dose of a Pre-S1/Pre-S2/S vaccine. J Hepatol 34(1):123–127

Shapiro AJ, Esther CR, Leigh MW, Dellon EP (2013) Vaccine induced Hepatitis A and B protection in children at risk for cystic fibrosis associated liver disease. Vaccine 31:906–911

Shchelkunov SN, Salyaev RK, Pozdnyakov SG, Rekoslavskaya NI, Nesterov AE, Ryzhova TS, Sumtsova VM, Pakova NV, Mishutina UO, Kopytina TV (2006) Immunogenicity of a novel, bivalent, plant-based oral vaccine against hepatitis B and human immunodeficiency viruses. Biotechnol Lett 28(13):959–967

Shivananda SV, Srikanth B, Mohan M, Kulkarni P (2006) Comparison of two hepatitis B vaccines (GeneVac-B and Engerix-B) in healthy infants in India. Clin Vaccine Immunol 13(6):661–664

Shukla A, Khatri K, Gupta PN, Goyal AK, Mehta A, Vyas SP (2008) Oral immunization against hepatitis B using bile salt stabilized vesicles (bilosomes). J Pharm Pharm Sci 11(1):59–66

Simard EP, Miller JT, George PA, Wasley A, Alter MJ, Bell BP, Finelli L (2007) Hepatitis B vaccination coverage levels among healthcare workers in the United States, 2002–2003. Infect Control Hosp Epidemiol 28(7):783–790

Sojikul P, Buehner N, Mason HS (2003) A plant signal peptide–hepatitis B surface antigen fusion protein with enhanced stability and immunogenicity expressed in plant cells. Proc Natl Acad Sci U S A 100(5):2209–2214

Stanford M, Whittall T, Bergmeier L, Lindblad M, Lundin S, Shinnick T, Mizushima Y, Holmgren J, Lehner T (2004) Oral tolerization with peptide 336–351 linked to cholera toxin B subunit in preventing relapses of uveitis in Behcet's disease. Clin Exp Immunol 137(1):201–208

Sun JB, Czerkinsky C, Holmgren J (2010) Mucosally induced immunological tolerance, regulatory T cells and the adjuvant effect by cholera toxin B subunit. Scand J Immunol 71(1):1–11

Sunil Kumar GB, Ganapathi T, Revathi C, Srinivas L, Bapat V (2005) Expression of hepatitis B surface antigen in transgenic banana plants. Planta 222(3):484–493

Suzuki K, Kaminuma O, Yang L, Takai T, Mori A, Umezu-Goto M, Ohtomo T, Ohmachi Y, Noda Y, Hirose S (2011) Prevention of allergic asthma by vaccination with transgenic rice seed expressing mite allergen: induction of allergen: specific oral tolerance without bystander suppression. Plant Biotechnol J 9(9):982–990

Tacket CO, Pasetti MF, Edelman R, Howard JA, Streatfield S (2004) Immunogenicity of recombinant LT-B delivered orally to humans in transgenic corn. Vaccine 22(31–32):4385–4389. doi:10.1016/j.vaccine.2004.01.073

Taddio A, Ipp M, Thivakaran S, Jamal A, Parikh C, Smart S, Sovran J, Stephens D, Katz J (2012) Survey of the prevalence of immunization non-compliance due to needle fears in children and adults. Vaccine 30(32):4807–4812

Takagi H, Hiroi T, Yang L, Tada Y, Yuki Y, Takamura K, Ishimitsu R, Kawauchi H, Kiyono H, Takaiwa F (2005a) A rice-based edible vaccine expressing multiple T cell epitopes induces oral tolerance for inhibition of Th2-mediated IgE responses. Proc Natl Acad Sci U S A 102(48):17525–17530

Takagi H, Saito S, Yang L, Nagasaka S, Nishizawa N, Takaiwa F (2005b) Oral immunotherapy against a pollen allergy using a seed-based peptide vaccine. Plant Biotechnol J 3(5):521–533

Thanavala Y, Mahoney M, Pal S, Scott A, Richter L, Natarajan N, Goodwin P, Arntzen CJ, Mason HS (2005) Immunogenicity in humans of an edible vaccine for hepatitis B. Proc Natl Acad Sci U S A 102(9):3378–3382

Tielemans CL, Vlasak J, Kosa D, Billiouw J-M, Verpooten GA, Mezei I, Ryba M, Peeters PC, Mat O, Jadoul MY (2011) Immunogenicity and safety of an investigational AS02v-adjuvanted hepatitis B vaccine in patients with renal insufficiency who failed to respond or to maintain antibody levels after prior vaccination: results of two open, randomized, comparative trials. Vaccine 29(6):1159–1166

Tohme RA, Awosika-Olumo D, Nielsen C, Khuwaja S, Scott J, Xing J, Drobeniuc J, Hu DJ, Turner C, Wafeeg T (2011) Evaluation of hepatitis B vaccine immunogenicity among older adults during an outbreak response in assisted living facilities. Vaccine 29:9316–9320

Van Damme P, Cramm M, Safary A, Vandepapeliere P, Meheus A (1992) Heat stability of a recombinant DNA hepatitis B vaccine. Vaccine 10(6):366–367

Vargas M, Montero C, Sánchez D, Pérez D, Valdés M, Alfonso A, Joglar M, Machado H, Rodríguez E, Méndez L (2010) Two initial vaccinations with the Bm86-based Gavacplus vaccine against *Rhipicephalus (Boophilus) microplus* induce similar reproductive suppression to three initial vaccinations under production conditions. BMC Vet Res 6(1):43

Velu V, Nandakumar S, Shanmugam S, Jadhav SS, Kulkarni PS, Thyagarajan SP (2007) Comparison of three different recombinant hepatitis B vaccines: Genevac B, Engerix B and Shanvac B in high risk infants born to HBsAg positive mothers in India. World J Gastroenterol 13(22):3084–3089

Venter C, Skypala I, Dean T (2008) Maize allergy: what we have learned so far. Clin Exp Allergy 38(12):1844–1846

Verma D, Moghimi B, LoDuca PA, Singh HD, Hoffman BE, Herzog RW, Daniell H (2010) Oral delivery of bioencapsulated coagulation factor IX prevents inhibitor formation and fatal anaphylaxis in hemophilia B mice. Proc Natl Acad Sci U S A 107(15):7101–7106

Vida Pérez L, Gómez Camacho F, García Sánchez V, Iglesias Flores EM, Castillo Molina L, Cerezo Ruiz A, Casáis Juanena L, De Dios Vega JF (2009) Eficacia de la vacuna contra el virus de la hepatitis B en pacientes con enfermedad inflamatoria intestinal. Med Clin 132(9):331–335

Vyas G, Rao K, Ibrahim A (1972) Australia antigen (hepatitis B antigen): a conformational antigen dependent on disulfide bonds. Science (New York, NY) 178(67):1300–1301

Wasley A, Kruszon-Moran D, Kuhnert W, Simard EP, Finelli L, McQuillan G, Bell B (2010) The prevalence of hepatitis B virus infection in the United States in the era of vaccination. J Infect Dis 202(2):192–201

Weinstein T, Chagnac A, Boaz M, Ori Y, Herman M, Zevin D, Schmilovitz-Weiss H, Gafter U (2004) Improved immunogenicity of a novel third-generation recombinant hepatitis B vaccine in patients with end-stage renal disease. Nephron Clin Pract 97(2):c67–c72

WHO (2010) WHO vaccine-preventable diseases: monitoring system 2010 global summary. Immunization, Vaccines and Biologicals

Williams RE, Sena AC, Moorman AC, Moore ZS, Sharapov UM, Drobenuic J, Hu DJ, Wood HW, Xing J, Spradling PR (2012) Hepatitis B vaccination of susceptible elderly residents of long term care facilities during a hepatitis B outbreak. Vaccine 30(21):3147–3150

Woo PC, L-p W, B-j Z, K-y Y (2001) Unique immunogenicity of hepatitis B virus DNA vaccine presented by live-attenuated *Salmonella typhimurium*. Vaccine 19(20):2945–2954

Wu JY, Newton S, Judd A, Stocker B, Robinson WS (1989) Expression of immunogenic epitopes of hepatitis B surface antigen with hybrid flagellin proteins by a vaccine strain of Salmonella. Proc Natl Acad Sci U S A 86(12):4726–4730

Wu Q, G-h Z, X-l W, T-j H, Shah DP, X-l W, L-r W, Zhang M (2012) Comparison of long-term immunogenicity (23 years) of 10 μg and 20 μg doses of hepatitis B vaccine in healthy children. Hum Vaccin Immunother 8(8):1071–1076

Yamamoto K, Horikita M, Tsuda F, Itoh K, Akahane Y, Yotsumoto S, Okamoto H, Miyakawa Y, Mayumi M (1994) Naturally occurring escape mutants of hepatitis B virus with various mutations in the S gene in carriers seropositive for antibody to hepatitis B surface antigen. J Virol 68(4):2671–2676

Yang L, Hirose S, Suzuki K, Hiroi T, Takaiwa F (2012) Expression of hypoallergenic Der f 2 derivatives with altered intramolecular disulphide bonds induces the formation of novel ER-derived protein bodies in transgenic rice seeds. J Exp Bot 63(8):2947–2959

Youm J-W, Won Y-S, Jeon JH, Ryu CJ, Choi Y-K, Kim H-C, Kim B-D, Joung H, Kim HS (2007) Oral immunogenicity of potato-derived HBsAg middle protein in BALB/c mice. Vaccine 25(3):577–584

Zhang Q, Zhong J, Huan L (2011) Expression of hepatitis B virus surface antigen determinants in *Lactococcus lactis* for oral vaccination. Microbiol Res 166(2):111–120

Zhao Z, Murphy TV, Jacques-Carroll L (2011) Progress in newborn hepatitis B vaccination by birth year cohorts-1998–2007, USA. Vaccine 30(1):14–20

Zheng BJ, Ng MH, Chan KW, Tam S, Woo PC, Ng SP, Yuen KY (2002) A single dose of oral DNA immunization delivered by attenuated Salmonella typhimurium down-regulates transgene expression in HBsAg transgenic mice. Eur J Immunol 32(11):3294–3304

Zitt E, Sprenger-Mähr H, Knoll F, Neyer U, Lhotta K (2011) Vitamin D deficiency is associated with poor response to active hepatitis B immunisation in patients with chronic kidney disease. Vaccine 30:931–935

Part III
Industrial Proteins

Chapter 12
Commercial Plant-Produced Recombinant Cellulases for Biomass Conversion

Elizabeth E. Hood and Deborah V. Requesens

12.1 Introduction to the Protein Product

Several fuel crises over the years, including those during the mid-1970s and mid-1980s, motivated research and production efforts into making fuel alternatives to oil. However, each time the urgency faded with the resurgence of inexpensive oil, the support for developing biofuels waned in parallel. In the late 1990s, a refocus of research to produce biofuels grew out of the Department of Energy, with a number of the national laboratories participating in developing and testing new technologies.

Recently, in an attempt to reduce dependence on foreign oil and address environmental, economic, and national security concerns, the production of biofuels has become a central topic once again. Roughly 40 percent of the US domestic corn production is used to produce ethanol. The remainder goes toward feed, food, and industrial use (USDA ERS, http://www.ers.usda.gov). Ethanol from corn starch has been available for 20 years and currently provides almost all the blended ethanol in gasoline in the USA. The 2012 production was over 13 billion gallons with the volume increasing yearly for the last decade. Much debate has occurred about the wisdom of making ethanol from corn because it is a food and feed commodity. Multiple articles have cited ethanol from corn as the driver for increasing feed prices and creating food shortages, making the use of corn to produce ethanol as a fuel source a controversial issue. However, current USDA data from the Economic Research Service show that productivity has increased along with usage of corn for ethanol and no increased cost for feed and food could be attributed to ethanol production (http://www.ers.usda.gov/publications/oce-usda-agricultural-projections/oce131.aspx).

Nevertheless, ethanol from corn starch is not able to generate enough biofuel to replace fossil fuel-derived gasoline because the volume of corn is not high enough. Thus, alternate sources of feedstock have been sought, creating interest in

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lignocellulosic plant biomass. Plant biomass comprises lignocellulose—a combination of cellulose, hemicellulose, pectin, and lignin. The advantage of producing biofuels from lignocellulose over other sources like corn and sugar cane is that the raw material is abundant and very diverse; in fact, availability of over a billion tons of biomass per year from agriculture and forestry production has been estimated in the USA (Perlack and Stokes 2011). This level of feedstock availability would provide adequate fuels to replace a significant amount of oil-based gasoline to generate energy independence and a secure fuel source. On the other hand, the biggest disadvantage of this biofuel source is that the technology necessary to process lignocellulose into the sugar monomers essential for fermentation into fuels is not mature enough to be cost-effective.

Cellulosic biofuels can be produced in a sustainable manner, in high volumes, and at low costs. However, gathering the feedstock, the initial pretreatment of cellulosic material, as well as the actual saccharification are expensive steps in the cellulose conversion process due to the low density and high complexity of the feedstock. Gathering the biomass, transporting it to the treatment location, and storing it for the allotted time needed until utilized have presented massive problems in logistics. On top of these issues, plant biomass, with all its complex molecules, is extremely difficult to break down into its components, and pretreatment is required to modify the structure and allow access for the enzymes to reach the macromolecules to produce sugars.

Intact sugars from cell wall macromolecules are required for fermentation into desired biofuels and bio-based products which can include ethanol but may also comprise butanol and other longer chain hydrocarbons, as well as chemical intermediates for manufacturing. The biochemical process usually includes a pretreatment step (Saville 2011) prior to deconstruction of the cellulose using cocktails of enzymes that specifically act on the substrate. The pretreatment is designed to make the highly structured cellulose more accessible to cellulases, but the process tends to destroy lignin structure and generate various inhibitors. Several types of pretreatment have been designed: AFEX (Garlock et al., 2012), steam explosion (Liu et al. 2013), and acid steam explosion (Chang et al. 2012; Gao et al. 2013), among others. An interesting pretreatment that preserves lignin structure uses ethanol to extract lignin as a valuable coproduct (Arato et al. 2005).

Once pretreatment of biomass has been completed, enzymes are added to the biomass to deconstruct the polysaccharides into sugars. Several major enzyme manufacturers have participated in developing enzyme cocktails for application to biomass. These companies include Novozymes, DuPont/Genencor, DSM, and Dyadic, among others. It is important to note that in order to produce the mandated 20 billion gallons of biofuel by year 2022 in the USA, two hundred thirty-five million dry tons of biomass would be needed. Based on estimates by the National Renewable Energy Lab (NREL) and current enzyme manufacturing recommendations, the total amount of enzyme required for 20 billion gallons of biofuel would be approximately 0.6 million tons. If these enzymes were to be produced by conventional fungal fermentation, the tanks and instrumentation alone for this much enzyme would cost nearly $30 billion before enzyme production processes even begin (Kameneva et al. 2011). While many of the above companies have made good progress toward lowering the cost of the

enzymes for biomass conversion, none has achieved low enough prices to enable industry development, as the cost of enzymes are estimated to range from $0.50 to $1.47 per gallon of ethanol equivalent (Klein-Marcuschamer et al., 2012). Alternatively, researchers from NREL, the US Department of Energy's primary national laboratory for renewable energy and energy efficiency R&D, are currently exploiting the synergy of two enzymatic systems to break down cell walls faster (Chundawat et al. 2011; Himmel et al. 2010). They have found that mixing free and complexed enzymes can enhance catalytic performance. Continual improvements are being made to fungal strains to accumulate higher levels of enzymes, but they are unlikely to be much greater than current levels because of the maturity of the technology.

Alternative approaches are needed to dramatically lower enzyme cost and provide the cellulases in large volumes. To address this, one can incorporate the enzymes into the biomass itself to lower its recalcitrance (Brunecky et al., 2011); Gray et al. 2011). A more universal system is to produce enzymes in the seed of a commodity crop such as corn (Hood et al. 2007).

12.2 Description of the Systems Used to Produce the Protein

Several problems are associated with a technology system based on microbial enzyme supply including the extremely large demand on infrastructure and the need to retain sterility during production. By contrast, an agricultural bio-production system offers the potential for a viable and scalable alternative to lower the cost of enzymes for biomass deconstruction. A seed-based production system will reduce the production cost and require a much lower capital investment because it would include only planting and harvesting equipment and it would be scalable to meet demands.

In contrast to microbial production, the technology curve for cellulase accumulation in plants is on the upswing with much room for improvement and optimization. In the near term, plant-produced hydrolytic enzymes may be formulated as crude aqueous extracts from plant materials—leaves, seeds, stalks, or their fractions (Sainz 2009). Cellulases have been expressed in a number of plant systems, primarily in the leaves and stems. The main advantage of a plant-based production system is the need for lower energy input than microbial production. It has nonetheless some challenges related to the heterologous expression of these bacterial and fungal enzymes in plants. In many cases codon alteration is necessary, and difficulties with expression levels are encountered. Several examples of successful expression can be cited, such as extracts of tobacco leaves expressing hyperthermophilic enzymes that showed accelerated glucose release from carbohydrate-rich substrates (Montalvo-Rodriguez et al. 2000). Oraby et al. (2007) demonstrated increased release of glucose from maize stover (22 %) and rice straw (30 %) over controls when expressing E1 endoglucanase from *Acidothermus cellulolyticus*. Other examples include *Arabidopsis thaliana* expressing E1, resulting in increased cell size and changes in cell wall structure (Park et al. 2003), potato producing E1 or xylanases (Dai et al. 2000;

Yang et al. 2007), barley overexpressing a fungal xylanase gene (Patel et al. 2000), or alfalfa plants producing bacterial cellulases (Ziegelhoffer et al. 1999).

In parallel to these academic studies, a number of companies are developing commercial plant lines. Enzymes involved in the degradation of lignocellulosic biomass can be produced in the plant to be used as feedstock. Agrivida is developing biomass engineered with pretreatment and cellulose-degrading traits, which are activated following harvest. They have developed engineered corn plants for lignocellulosic conversion using corn stover and cobs and also other dedicated feedstock for energy, such as switchgrass and sorghum. The plants contain enzymes involved in lignocellulosic conversion, such as xylanases and cellulases (Shen et al. 2012; Zhang et al. 2011).

Edenspace Systems Corporation (http://www.edenspace.com/home/biofuels/) has developed bioengineered plants with improved conversion properties, improving the production of renewable fuels from nonfood biomass sources such as poplar, corn stover, and switchgrass. Their goal is to reduce the cost of exogenous enzymes by more than 60 %. These examples of crops producing their own cell wall-degrading enzymes require characterization of each transformation event, which will require achieving nonregulated status of each plant type and will incur an extremely high cost and lengthy process prior to broad production (Sparrow et al. 2013).

An alternative method to whole plant enzyme expression employs the production of large quantities of enzymes in a seed production system. The approach used for enzyme expression described here is to overexpress the enzymes in seed tissue, thus enabling the plant to accumulate the protein in a specific plant part and allowing it to be applied to any other plant biomass source pretreated in any manner. Because this universal system utilizes seed, the enzymes can be stored for many years stably (Kusnadi et al. 1998). This long-term storability is likely due in part to the presence of protease inhibitors, the dry state of the seed, and the high concentration of carbohydrates that can stabilize proteins (Stoger et al. 2005). Utilizing corn lines to produce large quantities of cellulases in grain offers a realistic capability to meet the cost and scale requirements for cellulases on the current timeline for cellulosic biofuels.

12.3 Technical Progress

12.3.1 1. β-1,4 Glucanase, E1, a Thermostable Endocellulase

12.3.1.1 Protein Accumulation

The thermostable endoglucanase, E1 (Cel5A), from *Acidothermus cellulolyticus* has been expressed in many plants as described above. However, most of those systems were for demonstration purposes and not for production. The corn seed expression system is designed for large-scale production of the enzyme for industrial applications. Because the protein is thermostable, its stability is remarkable in most foreign expression systems. Thus, it was not surprising for the E1 protein to accumulate to levels of >15 % of total soluble protein (TSP) in T1 seed of several transgenic corn

lines (Hood et al. 2007). In order to achieve this high accumulation, we used a 1.4 kb upstream region of the maize globulin-1 promoter (Belanger and Kriz 1991) combined with targeting sequences that ensured protein accumulation in the apoplast (barley alpha amylase signal sequence, BAASS) (Rogers 1985), the endoplasmic reticulum (ER, KDEL), or the vacuole (Holwerda et al. 1992). These targeting sites have been the best locations for foreign protein expression in maize seed for a number of proteins (Streatfield et al. 2002; Hood et al. 2003; Woodard et al. 2003). However, it is not evident a priori which site will be best for any particular protein. Thus the protein of interest should be tested in each location. E1 expression is best in the vacuole and ER and, although present in the apoplast, this tissue appeared to be a less viable environment for this enzyme. All expression vectors also contained the protease inhibitor II (Pin II) terminator from potato (An et al. 1989).

In each vector, the E1 endoglucanase full-length gene was fused to the control sequences for expression. Several independent transgenic events (ITEs) and numerous plants per event were recovered from transformation of corn embryos using *Agrobacterium tumefaciens* (Hood et al. 2007). The ITEs were selected on the herbicide bialaphos using the maize-optimized phosphinothricin N-acetyltransferase gene (pat) from *Streptomyces viridochromogenes* (White et al. 1990). Individual seeds from each plant line were screened by enzyme activity assay, and plants with high protein accumulation in T1 seed were selected to move forward into a breeding program (Hood et al. 2012). These lines included some plants with vacuole-targeted protein (BCH) and some with ER-targeted protein (BCF). The breeding program has two goals: to improve the agronomic quality of the plants over the original transformation material and to improve the accumulation of recombinant protein in the seed. Several transgenic lines were backcrossed to elite inbreds for approximately three generations, and those with the best characteristics—growth and protein accumulation—were chosen for further breeding and characterization.

Transgenic lines BCH0101 and BCF0307 have been moved into production. Each of these ITEs contains a single insertion and copy of the gene (Hood et al. 2007). The backcross process requires 7 generations to move the transgenic trait into a 99 % elite inbred background. This backcross program is accomplished with two elite inbreds that when crossed produce a productive hybrid (Figs. 12.1 and 12.2). Because we also select for high protein accumulation, the elite inbreds have increasing amounts of the protein encoded by the transgene at each generation. Thus, the two elite inbreds that produce a high-yielding hybrid can now be self-pollinated to generate homozygous inbreds that produce the recombinant protein with two copies of the transgene. When these homozygous inbred lines are crossed to each other to produce hybrid seed, the hybrid seed contains a homozygous transgene, allowing maximal recombinant protein accumulation. We have shown that gene dosage positively influences protein accumulation in a dose-dependent manner (Hood et al. 2012). For the E1 recombinant protein, this is approximately 0.8 % of dry weight in the homozygous elite inbred line and in the hybrid seed and grain.

These inbred lines represent parent seed (homozygous parents of each side of a hybrid). The parents must be perpetuated as inbred lines, and the hybrids are produced from a single cross in one direction from the elite inbred parents (Fig. 12.2). In the case of E1 cellulase, the Stiff Stalk variety germplasm inbred parent, SP114 (USP

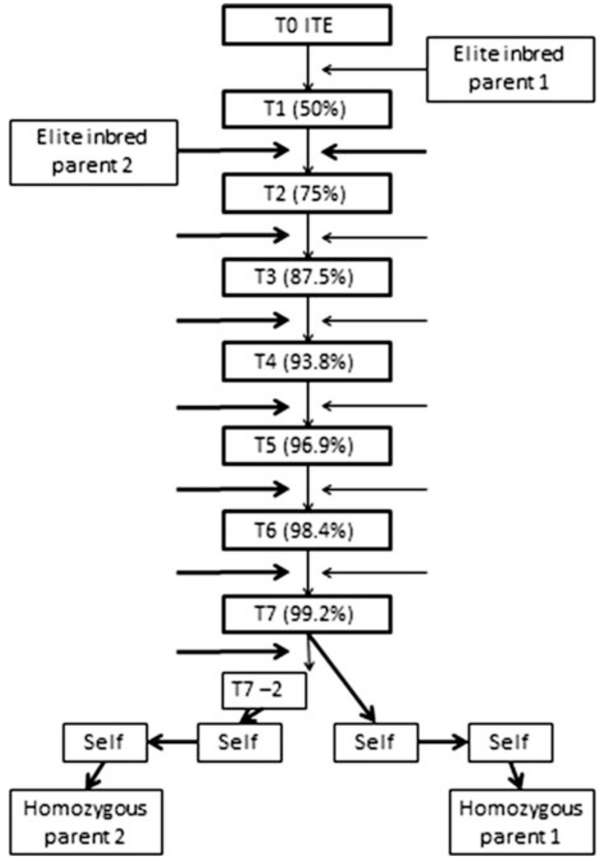

Fig. 12.1 Each independent transgenic event (ITE) recovered from tissue culture is the T0 generation. When this plant flowers in the greenhouse, the female ear is pollinated with pollen from a selected elite inbred (parent 1) that will be a parent of the hybrid for production. The seed that is recovered from this first cross is replanted, and half the herbicide-resistant individuals (this trait being linked to the rDNA protein of interest) are crossed with the second elite inbred (parent 2) and half are backcrossed to the parent 1 inbred. Each of these backcross schemes continues until seven generations for each have been recovered so that greater than 99 % of the genes in the lines are from the inbred and are coupled with the transgenic phenotype. The plants are then self-pollinated twice to recover homozygous inbred germplasm

#6,252,148), is the preferred female and the Lancaster variety, SP122 (USP #5,773,683), is the inbred male parent. Hybrid seed production requires that the female is sterile or de-tasseled and the male is able to cross-pollinate the female to produce hybrid seed. The hybrid seed is then used for grain production in the following season. The grain from the production fields is used for protein production whether for purified protein, concentrated extract, or defatted germ formulations.

The full-length gene not only contains the catalytic domain that cleaves internal bonds in the cellulose chain but has a cellulose-binding domain (CBD) that allows the enzyme to cling to the cellulose microfibril. The holoprotein is calculated at

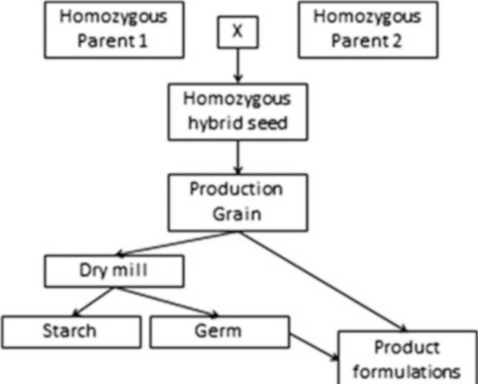

Fig. 12.2 Hybrid seed is produced from crosses of the two homozygous inbred parents. The hybrid seed is then planted to produce the grain that is used for enzyme production in various product formulations. Some formulations, such as purified protein, are most cost-effectively produced from whole grain to avoid the expense of dry milling. However, when producing bulk products, it is often more cost-effective to remove the starch for separate sales and retain the germ for enzyme formulations because the germ contains the entire recombinant protein complement of the grain

approximately 56.5 kDa with the catalytic domain at about 40 kDa (http://www.cazy.org/) (Cantarel et al. 2009). When this protein is extracted and characterized from maize seed, the protein is approximately 42 kDa and also very active, indicating that the catalytic domain is present but that the CBD may be missing (Hood et al. 2007). This could be confirmed through MALDI-TOF or protein sequencing. This phenomenon has been seen with most plant expression systems—the smaller, active domain is present but not the holoprotein. Fortunately, in the case of E1, this does not have a significant practical effect on its ability to deconstruct cellulose.

12.3.2 Cellobiohydrolase I, or CBHI, an Exocellulase

12.3.2.1 Protein Accumulation

An additional cellulase that has been expressed in corn seed is the cellobiohydrolase I (Cel 7A) gene from *Trichoderma reesei,* a filamentous fungus from which enzyme cocktails are being developed for applications in biomass conversion (Shoemaker et al. 1983). CBH I is an exocellulase that attacks the nonreducing free ends of the cellulose chain and cleaves off short oligomers of glucose. Similarly to E1, the full-length CBH I gene was fused to a 1.4 kb upstream fragment of the maize globulin-1 promoter, the same three signal sequences (ER, apoplast, and vacuole), and the Pin II terminator (Hood et al. 2007).

Several ITEs were recovered on bialaphos-containing medium through expression of the *pat* gene in these vectors. For CBH I, the best subcellular location for protein

accumulation was the apoplast, with >10 % total soluble protein in T1 seed from several ITEs (Hood et al. 2007). Although the enzyme accumulates in the cell wall space, no obvious plant damage was observed. This is likely because these enzymes are quite inefficient and require partners to digest cellulose (an endocellulase and β-glucosidase). The high level of expression was an unexpected and exciting result because previous reports for expression of CBH I in heterologous plant systems showed very low amounts of enzyme, less than 0.02 % TSP. ER-localized protein was active on soluble substrates and thus also highly accumulated, but protein in this location had lost the cellulose-binding domain, and unlike E1, this domain is critical for digesting macromolecular cellulose. These ER-targeted lines were therefore not pursued.

Several high-accumulation lines were moved into the breeding program. Of these, two lines were selected for continuation as product lines, BCC0206 and BCC0709. Each of these ITEs had a single insertion and copy number of the transgenes (Hood et al. 2007). After completion of the seven backcross generations and two self-pollinated generations, the accumulation of the CBH I protein is approximately 0.5 % of dry weight of seed in the inbred and hybrid lines.

These established inbred lines are, as with E1 cellulase, the parent seed (homozygous parents of each side of a hybrid). The parents are perpetuated as inbred lines, and the hybrids are produced from a single cross in one direction from the elite inbred parents. As with E1, the Stiff Stalk variety germplasm inbred parent, SP114 (USP #6,252,148), is the preferred female and the Lancaster variety, SP122 (USP #5,773,683), is the inbred male parent. Hybrid seed production requires that the female is sterile or de-tasseled, and the male is able to cross-pollinate the female to produce hybrid seed. The hybrid seed is then used for grain production in a subsequent season. The grain is used for protein production, whether for purified protein or defatted germ formulations.

12.4 Nontechnical Considerations

12.4.1 Production

12.4.1.1 E1 Production

Once parent and hybrid lines are established, grain production can be accomplished. In 2010, several thousand pounds of grain were produced on a few acres from hybrid seed generated in 2009. The grain was stored in large totes in a metal shed without temperature or humidity control for more than 2.5 years since harvest. Although the grain itself sustained some insect and rodent damage, the E1 content did not significantly change. Some of the grain has been ground whole to extract protein for purification. The thermostability of E1 to 81 °C suggests that heat treatment of the extract from corn flour could act as a purification step. Indeed, a 60 °C treatment removes approximately 30 % of the measured protein content. After concentrating this extract, it could be applied

directly to a GigaCap column that binds 95 % of the remaining proteins, allowing E1 to flow through as an essentially pure protein. This protein is stable in solution at −20 °C and 4 °C for several months and 1 month, respectively.

The grain may also be processed by dry milling to remove the germ (embryo) fraction from the starch-containing endosperm. This step allows the fractions to be used in separate processes, with sales into different markets. The starch can be used in the paper industry for sizing (http://www.ecosynthetix.com/biolatexr-technology/ecospherer/default.aspx) or in the ethanol industry for transportation fuels. The germ fraction in the case of E1-expressing corn contains the entire complement of the enzyme. Thus, the dry milling allows recovery of the E1 enzyme in a much smaller volume of production material. For stability, the oil should be removed from the germ to prevent oxidation and resulting rancidity. The defatted germ becomes the stored material with concentrated enzyme that can be used directly in industrial processes or ground and extracted for a liquid protein formulation.

Infinite Energy, LLC (d.b.a. Infinite Enzymes) is commercializing formulations of E1 and CBH I from transgenic corn grain. Each enzyme can be efficiently extracted using an acidic low-salt buffer. This low-salt buffer extracts less native corn protein than neutral buffers, allowing early partial purification. The E1 endocellulase is precipitated in ammonium sulfate (AS), desalted, and because the E1 is highly heat stable, the mixture is heat treated at 60 °C to remove corn protein. The resulting solution is subjected to column chromatography. The protein is 95 % pure at this stage. E1 endocellulase is also produced as a concentrated extract (approximately 7× concentrated) from corn grain extracted in acidified water.

Purified E1 enzyme and concentrated extracts are both active in digesting cellulosic materials (Fig. 12.3; unpublished results). As a third formulation, Infinite Enzymes is using defatted germ (see dry milling discussion below) in direct addition to biomass deconstruction processes. Because the germ also contains cellulose that can contribute to the sugar stream recovered, the enzyme-containing germ has a higher value than just the enzymes alone (Kameneva et al. 2011).

The current production acres are being grown under permit because this transgenic line has not achieved nonregulated status. However, when considering the cost of collecting data for a petition for nonregulated status versus the cost of containment, several hundred acres can be grown under permit before the cost of the petition makes economic sense. Certainly for the use of E1 in the laboratory reagent market, the world market could be supplied with 1–2 acres of production. Applications in the pulp and paper market likely could be met with 100–1,000 acres, still feasible under permit. However, when enzyme is to be applied to the biomass conversion market, over 1 million acres would be required, thus triggering the need for nonregulated status.

12.4.1.2 CBH I Production

CBH I grain has been produced under permit on a few acres. Some of this grain was ground and extracted for purification of the CBH I protein. The protocol includes two ammonium sulfate precipitation steps (AS) to precipitate and concentrate the CBH I

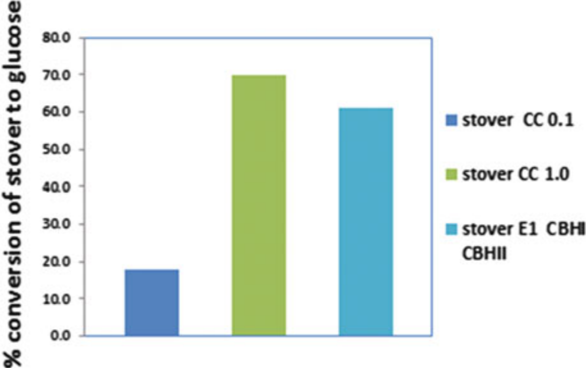

Fig. 12.3 Activity of corn seed-produced enzymes on pretreated corn stover samples. Pretreated corn stover samples were treated at 50 °C for 4 days with a commercial cellulase mixture at two concentrations (CC1 and CC10; Celluclast and additional enzymes purchased from Sigma Chemical Co.) demonstrating the release of glucose. Plant-produced enzymes (purified CBH I and E1 along with a concentrated sodium acetate extract of corn seed containing CBH II) were also very effective in releasing glucose from stover with only the addition of commercial β-glucosidase, demonstrating that the plant enzymes can be used in bioconversion. Digestions were performed at 50 °C for 4 days

enzyme. The AS pellet is dissolved in low salt, concentrated and purified by column chromatography. The combined fractions are buffer exchanged and then bound to an anion exchange resin (Hood et al. unpublished). The final product is greater than 95 % pure as viewed on a Coomassie blue-stained acrylamide gel. This product is being distributed through Sigma Chemical Company (St. Louis, MO) as E6412. The current product is stabilized with ammonium sulfate although lyophilization is also being tested.

Purified CBH I and concentrated extracts are active in biomass deconstruction (Hood et al. 2012). However, neither of these formulations is likely to be an inoculum for biomass conversion because of the added cost of extraction and/or purification. Thus, we are focusing on the defatted germ as a product for biomass conversion in addition to the purified enzyme for reagent applications. Dry milling of CBH I and E1 grain was accomplished at the National Corn-to-Ethanol Research Center in Edwardsville, IL. The recovered germ was defatted using a press (K. Humphrey, personal communication), although hexane extraction is also an option. The enzyme in defatted germ is stable for extended periods of time at 4 °C. The defatted germ formulation can be directly added to industrial applications.

12.5 Regulatory Permits: Planting Requirements

The largest barrier plant-based production of enzyme encounters is the regulatory process necessary for production. Most crop developers cannot afford the high cost of regulatory approval for GE crops. To be commercialized and grown without restrictions, GE crops must go through an extensive food, feed, and environmental

safety assessment process. Estimates of the cost of meeting regulatory requirements for GE crops range from $20–30 million (McElroy 2003) to over $100 million (Sparrow et al. 2013). Alternatively to this expensive process to achieve nonregulated status, a developer can obtain an authorization from the US Department of Agriculture, through either the permit process or notification process, to field test or release the GE plant into the environment in small plots of up to 100 to 1000 acres with isolation from other corn crops.

Alternative approaches to producing cellulases in plants encompass putting the enzyme into the biomass directly. Two approaches have been taken, putting the wild-type enzyme into biomass and putting an altered inactive form into biomass. Each of these approaches produces biomass that has improved deconstruction logistics. Each of these approaches also produces a product that will have to be petitioned for nonregulated status for the acres grown for dedicated biomass conversion.

12.6 Equipment

When using a commodity crop for production of a commodity, such as an industrial enzyme, the equipment required for production and processing includes that which is already utilized and familiar. For example, the only equipment required for the production of cellulase comprises plants, combines, sprayers, dryers, seed cleaners, degermers, and deoilers—readily available equipment for the commodity corn industry. Thus, expensive infrastructure is not necessary.

12.7 Public Perception

The advantages of using a commodity crop, such as maize, to produce cellulases for biomass conversion are numerous. Because the enzymes are produced in a crop, they can be grown near the biomass that will be converted into sugars for bio-based products. Thus, transportation costs for the enzymes should be minimal. The crop-based system can provide massive quantities of enzymes without added infrastructure because they are planted in soil and do not require tanks for growth. The enzymes are expressed in the germ of the maize kernel and thus can be integrated with acreage that is already dedicated to ethanol production. The starch from the endosperm of the kernel can be separated by dry milling for ethanol, and the germ containing the enzymes then becomes a coproduct of corn-to-ethanol production. Thus, the acreage dedicated to enzyme production does not impact additional acres over and above those for ethanol production from starch. Assuming saccharification requires 30 g enzyme per gallon, the number of acres necessary to produce enzymes for a 50 million gallon per year (MGY) biofuels plant is approximately 40,000 if the expression level is 1.0 % of dry weight of the kernel. Forty thousand acres is an area less than 64 square miles, or approximately an 8-mile square production area.

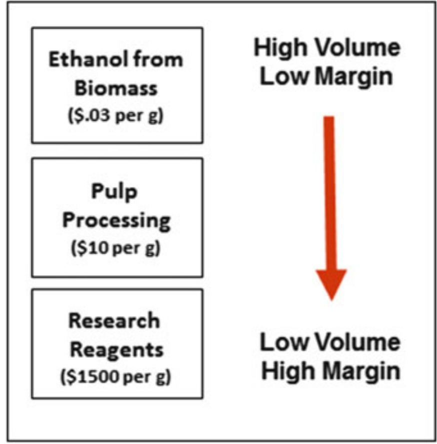

Fig. 12.4 Market analysis for cellulase enzymes. Multiple market applications are available for cellulases from any system. Each market has different volume requirements and margin of sales versus cost of goods

12.8 Conclusions

Plant-produced cellulases can achieve the cost targets required to make commodity chemicals from lignocellulosic substrates. In planta enzymes can lower the recalcitrance of the biomass to allow the seed-based enzymes to be more effective, maximizing the plant-based production system. Once a demonstration of the seed-based enzymes at pilot scale has been achieved, it will be obvious that this is a cost-competitive production system. Improving expression (accumulation) of the enzymes in the tissues, or stacking of the enzymes in a single seed, will improve cost structure. Any or all biomass deconstruction enzymes and accessory proteins, including ligninases in particular, would be excellent targets for the seed system. Much more information will be required to determine if stacking of enzymes in biomass tissue itself will be detrimental to the plant tissue.

The biomass to biofuels enzymes must be the lowest cost enzymes available because industry manufactures a commodity from a commodity. Nevertheless, because biofuels are a commodity, the volume of that market is huge, allowing the manufacturer to make revenue on the large volumes. However, because the biomass to biofuels markets are just developing, plant-based enzyme producers are addressing other markets first to establish sales (Fig. 12.4). Although these early markets are not large in comparison to the biofuels market, the enzymes can demand higher margins and higher prices. Purified endo- and exocellulases can be obtained through distributors' reagent chemical distributors. A concentrated extract of the endocellulase may be applied in recycled pulp processing. This application could be the first large-scale application of a plant-produced enzyme. We have demonstrated that the enzymes work competitively on a performance as well as on a cost basis and the remaining effort is to establish a supply chain.

Plant-produced enzymes offer a new supply that allows enzyme production without major steel infrastructure. The genetically engineered crop will have to undergo regulatory approval in order to achieve large-scale production acreage, adding an up-front cost to scale-up. However, even if the cost of approval is $20

million, it is a one-time cost that is still within the dollar realm of fermentation apparati. Thus, continued scale-up would be at no added cost.

12.9 Future Directions

The most important factor for producing commodity chemicals at a low cost is having a high concentration of the target chemical in the raw production material. Thus, future work will in part concentrate on an increase in expression of the recombinant protein. Various approaches include improving the molecular constructs to include appropriate targeting sequences and highly transcribed promoters (Streatfield 2007). In addition, new genes for cellulases that have higher activity can be transformed into the system for better cost-effectiveness. In either case, the recombinant protein accumulation in the initial transgenic event can be increased through genetic backcrosses to an elite inbred line with selection for higher expression levels.

The mechanism of the increases in recombinant protein accumulation within the backcrosses is not understood. At each backcross generation of a single genetic line, the ears segregate for accumulation of the recombinant protein. For example, the progeny from the T4 generation, including approximately 47 ears, were analyzed on a single ear basis. Fifty seeds were randomly chosen from the ear, ground, and 100 mg of the meal was extracted and analyzed for content of recombinant enzyme. Thus, the quantity of enzyme as a percent of dry weight can be determined (Fig. 12.5). One result observed is that the amount of target protein varies over a 5-fold range. Because these progenies are all from a single-parent plant, the transgene does not vary, but only the inheritance and interaction of the inbred parent genes with the locus of the transgene. Recent studies have demonstrated that corn has massive variability even within an inbred. Certainly these inbreds have the potential to inherit small variability that has a large impact on certain traits. Experiments are currently being conducted to understand the genetic factors that differentiate high- and low-expressing lines. Transcriptome analysis can be utilized to analyze near isogenic lines that show high and low expression to identify genes that control this phenomenon. Future experiments will focus on utilizing the identified genes to direct breeding to select lines at early stages of inheritance that promote increased protein accumulation. Genes that contribute to the quantitative inheritance of this phenomenon will have molecular markers identified that co-segregate with them. These markers can be then used to identify the desired traits in seedlings. This method should shorten the time to recover and assist in the selection of the high-expressing lines for production.

Additional work is required to understand the performance of the plant-produced enzymes in biomass conversion reactions at pilot scale. The performance at high concentrations of biomass in solution has only been done in small volumes (Fig. 12.3). The results show that the three plant-produced enzymes, E1, CBH I, and CBH II, work in concert with β-glucosidase to digest corn stover to the same

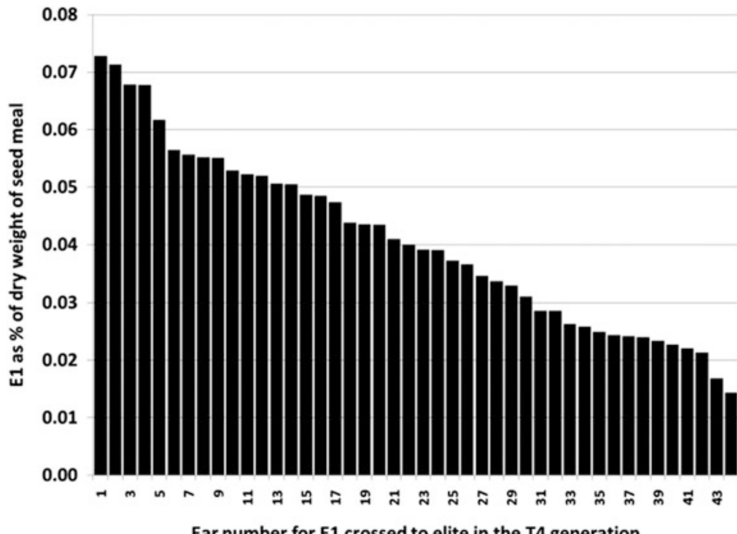

Fig. 12.5 The progeny from a single E1 transgenic plant from a single independent transgenic event (BCH0101, vacuole-targeted protein) segregate in content of recombinant protein. Each bar on the graph represents the results from a single ear analysis

degree as commercial cellulase preparations at approximately equal protein-loading levels. Although larger volume testing is in process, commercialization will require testing at volumes of at least 1,000 L to ensure efficacy.

References

An G, Mitra A, Choi HK, Costa MA, An K, Thornburg RW, Ryan CA (1989) Functional analysis of the 3[prime] control region of the potato wound-inducible proteinase inhibitor ii gene. Plant Cell Online 1(1):115–122. doi:10.1105/tpc.1.1.115

Arato C, Pye EK, Gjennestad G (2005) The lignol approach to biorefining of woody biomass to produce ethanol and chemicals. Appl Biochem Biotechnol 123:871–882

Belanger F, Kriz A (1991) Molecular basis for allelic polymorphism of the maize globulin-1 gene. Genetics 129(3):863–872

Brunecky R, Selig M, Vinzant T, Himmel M, Lee D, Blaylock M, Decker S (2011) In planta expression of A. cellulolyticus Cel5A endocellulase reduces cell wall recalcitrance in tobacco and maize. Biotechnol Biofuels 4:1

Cantarel BL, Coutinho PM, Rancurel C, Bernard T, Lombard V, Henrissat B (2009) The Carbohydrate-Active EnZymes database (CAZy): an expert resource for glycogenomics. Nucleic Acids Res 37(Database issue):D233–D238. doi:10.1093/nar/gkn663

Chang J, Cheng W, Yin Q, Zuo R, Song A, Zheng Q, Wang P, Wang X, Liu J (2012) Effect of steam explosion and microbial fermentation on cellulose and lignin degradation of corn stover. Bioresour Technol 104:587–592

Chundawat SP, Beckham GT, Himmel ME, Dale BE (2011) Deconstruction of lignocellulosic biomass to fuels and chemicals. Annu Rev Chem Biomol Eng 2:121–145

Dai Z, Hooker BS, Anderson DB, Thomas SR (2000) Expression of Acidothermus cellulolyticus endoglucanase E1 in transgenic tobacco: biochemical characteristics and physiological effects. Transgenic Res 9(1):43–54

Gao X, Kumar R, DeMartini JD, Li H, Wyman CE (2013) Application of high throughput pretreatment and co-hydrolysis system to thermochemical pretreatment. Part 1: Dilute acid. Biotechnol Bioeng 110:754–762

Garlock RJ, Bals B, Jasrotia P, Balan V, Dale BE (2012) Influence of variable species composition on the saccharification of AFEX™ pretreated biomass from unmanaged fields in comparison to corn stover. Biomass Bioenergy 37:49–59

Gray BN, Bougri O, Carlson AR, Meissner J, Pan S, Parker MH, Zhang D, Samoylov V, Ekborg NA, Michael Raab R (2011) Global and grain-specific accumulation of glycoside hydrolase family 10 xylanases in transgenic maize (Zea mays). Plant Biotechnol J 9(9):1100–1108

Himmel ME, Xu Q, Luo Y, Ding S-Y, Lamed R, Bayer EA (2010) Microbial enzyme systems for biomass conversion: emerging paradigms. Biofuels 1(2):323–341

Holwerda C, Padgett H, Rogers J (1992) Proaleurain vacuolar targeting is mediated by short contiguous peptide interactions. Plant Cell 4(3):307–318

Hood EE, Bailey MR, Beifuss K, Magallanes-Lundback M, Horn ME, Callaway E, Drees C, Delaney DE, Clough R, Howard JA (2003) Criteria for high-level expression of a fungal laccase gene in transgenic maize. Plant Biotechnol J 1(2):129–140. doi:10.1046/j.1467-7652.2003.00014.x, PBI014 [pii]

Hood E, Love R, Lane J, Bray J, Clough R, Pappu K, Drees C, Hood K, Yoon S, Ahmad A (2007) Subcellular targeting is a key condition for high-level accumulation of cellulase protein in transgenic maize seed. Plant Biotechnol J 5(6):709–719

Hood EE, Devaiah SP, Fake G, Egelkrout E, Teoh K, Requesens DV, Hayden C, Hood KR, Pappu KM, Carroll J, Howard JA (2012) Manipulating corn germplasm to increase recombinant protein accumulation. Plant Biotechnol J 10:20–30. doi:10.1111/j.1467-7652.2011.00627.x

Kameneva T, Grayden DB, Meffin H, Burkitt AN (2011) Simulating electrical stimulation of degenerative retinal ganglion cells with bi-phasic pulse trains. Conf Proc IEEE Eng Med Biol Soc 2011:7103–7106. doi:10.1109/IEMBS.2011.6091795

Klein-Marcuschamer D, Oleskowicz-Popiel P, Simmons BA, Blanch HW (2012) The challenge of enzyme cost in the production of lignocellulosic biofuels. Biotechnol Bioeng 109:1083–1087

Kusnadi AR, Hood EE, Witcher DR, Howard JA, Nikolov ZL (1998) Production and purification of two recombinant proteins from transgenic corn. Biotechnol Prog 14(1):149–155

Liu Z-H, Qin L, Jin M-J, Pang F, Li B-Z, Kang Y, Dale BE, Yuan Y-J (2013) Evaluation of storage methods for the conversion of corn stover biomass to sugars based on steam explosion pretreatment. Bioresour Technol 132:5–15

McElroy D (2003) Sustaining agbiotechnology through lean times. Nat Biotechnol 21:996–1002

Montalvo-Rodriguez R, Haseltine C, Huess-LaRossa K, Clemente T, Soto J, Staswick P, Blum P (2000) Autohydrolysis of plant polysaccharides using transgenic hyperthermophilic enzymes. Biotechnol Bioeng 70(2):151–159

Oraby H, Venkatesh B, Dale B, Ahmad R, Ransom C, Oehmke J, Sticklen M (2007) Enhanced conversion of plant biomass into glucose using transgenic rice-produced endoglucanase for cellulosic ethanol. Transgenic Res 16(6):739–749

Patel M, Johnson JS, Brettell RI, Jacobsen J, Xue G-P (2000) Transgenic barley expressing a fungal xylanase gene in the endosperm of the developing grains. Mol Breed 6(1):113–124

Perlack RD, Stokes BJ (2011) US billion-ton update: biomass supply for a bioenergy and bioproducts industry. Oak Ridge National Laboratory, Oak Ridge, TN

Rogers JC (1985) Two barley alpha-amylase gene families are regulated differently in aleurone cells. J Biol Chem 260(6):3731–3738

Sainz M (2009) Commercial cellulosic ethanol: The role of plant-expressed enzymes. In Vitro Cell Dev Biol Plant 45(3):314–329

Saville B (2011) Pretreatment options. In: Hood EE, Nelson P, Powell R (eds) Plant biomass conversion. Wiley-Blackwell, Ames, IA
Shen B, Sun X, Zuo X, Shilling T, Apgar J, Ross M, Bougri O, Samoylov V, Parker M, Hancock E (2012) Engineering a thermoregulated intein-modified xylanase into maize for consolidated lignocellulosic biomass processing. Nat Biotechnol 30(11):1131–1136
Shoemaker S, Schweickart V, Ladner M, Gelfand D, Kwok S, Myambo K, Innis M (1983) Molecular cloning of exo-cellobiohydrolase I derived from Trichoderma reesei strain L27. Bio/Technology 1(8):691–696
Sparrow P, Broer I, Hood EE, Eversole K, Hartung F, Schiemann J (2013) Risk assessment and regulation of molecular farming-a comparison between Europe and US. Curr Pharm Des 19(5513–5530):1–18
Stoger E, Ma JK, Fischer R, Christou P (2005) Sowing the seeds of success: pharmaceutical proteins from plants. Curr Opin Biotechnol 16(2):167–173. doi:10.1016/j.copbio.2005.01.005
Streatfield SJ (2007) Approaches to achieve high-level heterologous protein production in plants. Plant Biotechnol J 5:2–15
Streatfield S, Mayor J, Barker D, Brooks C, Lamphear B, Woodard S, Beifuss K, Vicuna D, Massey L, Horn M, Delaney D, Nikolov Z, Hood E, Jilka J, Howard J (2002) Development of an edible subunit vaccine in corn against enterotoxigenic strains of Escherichia coli. In Vitro Cell Dev Biol Plant 38(1):11–17. doi:10.1079/ivp2001247
White J, Chang SY, Bibb MJ, Bibb MJ (1990) A cassette containing the bar gene of Streptomyces hygroscopicus: a selectable marker for plant transformation. Nucleic Acids Res 18:1062
Woodard S, Mayor J, Bailey M, Barker D, Love R, Lane J, Delaney D, McComas-Wagner J, Mallubhotla H, Hood E (2003) Maize (Zea mays)-derived bovine trypsin: characterization of the first large-scale, commercial protein product from transgenic plants. Biotechnol Appl Biochem 38(2):123–130
Yang P, Wang Y, Bai Y, Meng K, Luo H, Yuan T, Fan Y, Yao B (2007) Expression of xylanase with high specific activity from Streptomyces olivaceoviridis A1 in transgenic potato plants (Solanum tuberosum L.). Biotechnol Lett 29(4):659–667
Zhang D, VanFossen AL, Pagano RM, Johnson JS, Parker MH, Pan S, Gray BN, Hancock E, Hagen DJ, Lucero HA (2011) Consolidated pretreatment and hydrolysis of plant biomass expressing cell wall degrading enzymes. BioEnergy Res 4(4):276–286
Ziegelhoffer T, Will J, Austin-Phillips S (1999) Expression of bacterial cellulase genes in transgenic alfalfa (Medicago sativa L.), potato (Solanum tuberosum L.) and tobacco (Nicotiana tabacum L.). Mol Breed 5(4):309–318

Chapter 13
Brazzein: A High-Intensity Natural Sweetener

Gina Fake and John Howard

13.1 Introduction

Since the 1980s, overweight and obesity rates have been steadily increasing worldwide. Obesity is now the fifth leading risk of death, contributing to 44 % of the diabetes burden, 23 % of the heart disease burden, and between 7 and 41 % of certain cancer burdens (http://www.who.int/mediacentre/factsheets/fs311/e). Consequently, healthcare costs to treat obesity-related illnesses have risen as well. Worldwide, childhood obesity is at an all-time high with over 40 million overweight children under the age of five in 2010 (http://www.who.int/mediacentre/factsheets/fs311). In 2010, US First Lady Michelle Obama started the *Let's Move*! initiative to combat childhood obesity. As one of the five pillars of the initiative, improving access to healthy and affordable food, one recommendation was "food, beverage and restaurant industries should be encouraged to use their creativity and resources to develop or reformulate more healthful foods for children and young people" (http://www.letsmove.gov/sites/letsmove.gov/files/TFCO_Summary_of_Recommendations.pdf).

To meet consumer demand and political pressure for more healthful alternatives, the food and beverage industry has developed a range of products to appeal to the health-conscious. One of the largest market growth areas has been in artificial sweeteners [for a general review, see Priya and Gupta (2011), Nabors (2012)]. Since the 1980s, the market for artificial sweeteners has grown to more than 1 billion dollars a year. In that time, five artificial sweeteners have received FDA approval. That group—acesulfame K, aspartame, neotame, saccharin, and sucralose—has flooded supermarkets in products ranging from infant formula to soft drinks, chewing gum to bread. While production and use of these low-calorie artificial sweeteners has likely helped slow the obesity rate, the trend toward obesity continues steeply upward every

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year. Hurdles remain to meet full consumer acceptance of these nonsugar substitutes. Two key factors are concerns over perceived safety and taste.

As use of artificial sweeteners becomes more widespread, consumer concern for the safety of these substances has grown. The internet is rife with speculation of links between artificial sweeteners and a myriad of health issues such as cancer, premature birth, weight gain, and increased risk of metabolic syndrome. Though not typically backed by scientific evidence to support them, these claims fuel the fear of artificial sweeteners (http://www.nytimes.com/2014/01/05/magazine/the-quest-for-a-natural-sugar-substitute.html?emc=eta1&_r=0; http://www.livestrong.com/article/226677-what-are-the-dangers-of-artificial-sweeteners-in-diet-drinks). These reports have served to shake the consumer's sense of security regarding these products because many originate in nonfood materials.

Additionally, taste remains a driving force in consumer acceptance of any food product. Each of the synthetic sweeteners has different gustatory properties, and none is identical to sucrose. Two important characteristics evaluated under sensory testing are onset of sweetness and aftertaste. Both acesulfame k and saccharin are typically reported to have a rapid sweetness onset whereas neotame and sucralose have slower onset of sweetness compared to sucrose. Acesulfame K, saccharin, and sucralose have a bitter-metallic aftertaste, while neotame has a licorice-like cooling mouth effect. Consumer acceptance hinges on whether these sweetness characteristics are satisfactory.

13.2 Introduction to Natural Sweeteners

Most recently, the food and beverage industry has turned its attention to naturally sourced, high-intensity sweeteners. Because some of these natural sweeteners have long histories of consumption in their native plant, safety concerns are reduced. Some have even been given GRAS status in the USA, fast-tracking commercialization (e.g., Stevia FDA approval in 2008). However, as with artificial sweeteners, taste remains an obstacle for widespread consumer use and the search for a natural high-intensity sweetener with a better taste profile continues. Finally, cost is also an important factor for these products compared to the cost of synthetic sweeteners or sucrose.

The natural sweetener on the market currently is stevioside, which is derived from the extract of the native South American plant *Stevia rebaudiana* (Goyal and Goyal 2010). This first obtained acceptance in Japan and was more recently introduced into the USA. Its success has spurred interest in other natural sweeteners. These include a limited number of natural proteins that have been identified as having intense sweetness. Commercial application of these proteins in the past has been limited both by cost of production and sensory quality. Thaumatin is a natural sweet protein that can be obtained as an extract from the West African plant *katernfe* or produced as a recombinant protein. Thaumatin has been approved as a flavor enhancer in the USA, and the product is sold under the brand name *Talin* (Gibbs et al. 1996). However, thaumatin has limited market potential because its flavor perception as a sweetener is unacceptable for most food applications and its costs are relatively high. Nevertheless,

thaumatin provides an important regulatory precedent as it was granted GRAS status based on its history of safe use in Africa.

13.2.1 Brazzein, a Sweet Protein

A front-runner of the natural protein sweeteners is brazzein, found in the fruit of the African plant *Pentadiplandra brazzeana* (Ming and Hellekant 1994). With an intrinsic sweetness 500–2,000 times that of sucrose, brazzein is a good choice for a high-intensity alternative. While proteins and sugars have approximately the same caloric value on a weight basis, the fact that brazzein is approximately 1,000-fold sweeter than sucrose makes it a low-calorie option.

A summary of some of the sweeteners (Table 13.1), highlighting the key characteristics, is shown below. While not a commercial product yet, brazzein is included to highlight where it may be able to satisfy some unmet needs in the sweetener market.

Three different forms of the brazzein protein exist in nature, differing from one another at the N-terminal amino acid residue (Fig. 13.1). Type 2 brazzein is a 54-amino acid peptide with glutamine at its N-terminus. Type 1 brazzein results through the natural, non-catalytic conversion of the terminal glutamine to pyroglutamate, and the loss of the N-terminal glutamine or pyroglutamate yields Type 3 brazzein. Only Type 1 and Type 3 brazzein are detected in ripe fruit (Hellekant and Danilova 2005).

Early studies on the properties of brazzein were conducted at the University of Wisconsin-Madison (Hellekant and Danilova 2005). Their research used a sample of *P. brazzeana* pulp, but limited availability of the fruit meant another method of production was necessary. Use of a recombinant *Escherichia coli* production system allowed further characterization of the biochemical properties of brazzein, but low expression levels in the bacteria greatly limited research for protein stability and gustatory tests.

Experiments using yeast as an alternative expression system showed that it was capable of expressing brazzein at sufficient concentrations to allow purification of enough material to carry out pilot-scale operations (Howard, unpublished results). Using this system, brazzein was produced for initial sensory and stability testing. The conclusion of these experiments was that brazzein had the sensory attributes that could lead to a commercial product (unpublished results).

When the gene coding for Type 2 brazzein was expressed in yeast, it was unstable and non-catalytically converted to Type 1. Since Type 1 is not as sweet as Type 2, it presents a challenge for the stability of sweetness. One solution is to allow most of the Type 2 brazzein to convert to Type 1 before its use in food applications. This option, however, effectively lowers the sweetness potential and increases the cost. Type 3 brazzein, in contrast, did not undergo any chemical rearrangements. In addition to its stability, it is the sweetest of the three types of brazzein making it the preferred choice for commercialization.

The Type 3 gene was also expressed successfully in yeast, and a product with stable sweetness intensity was obtained. Scale-up to pilot production allowed the

Table 13.1 Comparison of high-intensity sweeteners

	High-intensity sweeteners						
	Saccharin	Cyclamates	Aspartame	Acesulfame K	Sucralose	Stevioside	Brazzein
Product source	Synthetic chemical	Synthetic chemical	Synthetic dipeptide	Synthetic chemical	Synthetic chemical	Plant extract	Plant protein
Mkt price[a]	+	++	+++	++++	+++	++++	?
Potency[b]	300 ×	30 ×	180 ×	200 ×	500 ×	200 ×	1,000 ×
FDA approval	GRAS	Removed	1981	1988	1998	2008	No
Key benefit	Cost	Cost	Better taste	Quick onset	Stability	Natural	?
Key issue[c]	Health	Health	Stability	Aftertaste	Health	Aftertaste	?

Attributes of common sweeteners
[a]Relative cost
[b]Sweetness relative to sucrose
[c]Perceived issue (not necessarily based on scientific data)
[?]Indicates unknown at this time

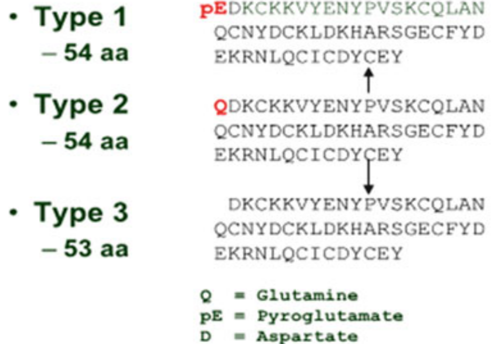

Fig. 13.1 Natural forms of brazzein. The N-terminal sequences of the three natural forms of brazzein

production of Type 3 brazzein purified to homogeneity for use in stability and sensory panels (Howard, unpublished results).

13.2.2 Sensory Panels Using Brazzein

Purified yeast-derived brazzein was used in a number of sensory experiments demonstrating that it was up to 2,000 times sweeter than sucrose. In taste panels, it was also described as having a “cooling taste.” When brazzein was blended with several other sweeteners, the results were a more sugar-like taste. Of particular note was the ability of brazzein to blend with sucrose to achieve a highly desirable taste profile with a lower overall sucrose level (Howard, unpublished results).

This latter observation was expanded on with a larger, nonexpert, taste trial. Panelists were presented with unmarked samples and asked to rank them based on a variety of characteristics including sweetness and overall taste preference. In two separate sensory panels, volunteer tasters were presented with four samples: (1) plain commercial yogurt, (2) yogurt with added sugar, (3) yogurt with added purified Type 3 brazzein, or (4) yogurt with a combination of sugar and brazzein. When asked to rank the samples for taste preference, the sugar + brazzein sweetened yogurt was chosen as the top sample in both panels (Fig. 13.2). Other characteristics, such as sweetness, were also rated and gave similar results (Howard, unpublished results).

13.3 Expression of Brazzein in Maize

The yeast system allowed for ample material to be used to develop purification protocols with the hope that it would be a practical production system. However, cost models (Howard, unpublished results) revealed that the yeast expression system under the conditions tested could not accumulate brazzein at levels that

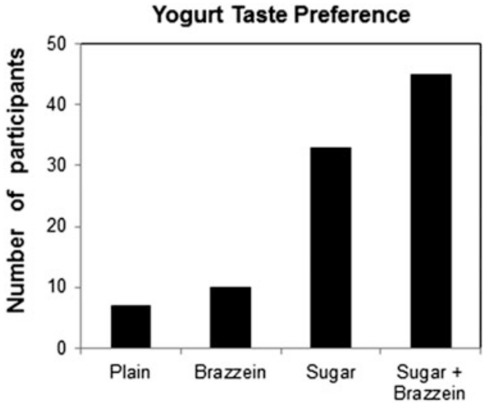

Fig. 13.2 Brazzein nonexpert taste panel. A double-blind experiment was carried out using yogurt with and without added sugar or brazzein. The results of the overall preference are shown below for the various yogurt samples. The number of people who preferred each type of yogurt is shown for one of the tests

would meet commercial cost targets. Therefore, a plant expression system was investigated as an alternative to microbial production. Maize was selected due to its low cost and abundance in the food chain.

Both Type 2 and Type 3 brazzein were initially engineered into maize and showed promising results with respect to expression level (Lamphear et al. 2005). Because of the desire to commercialize the product, later studies were done exclusively with the sweeter, more stable Type 3 brazzein. The maize-produced brazzein was identical to that of the yeast-produced brazzein. Following up on this early work, more constructs were made using stronger seed promoters. Recombinant plants obtained from these constructs were put through selection and backcrossing programs to yield material that accumulated up to 0.5 g brazzein per kg seed (Howard, unpublished results). These lines have not yet been fully optimized, and the expectation is that they will eventually be well above the 1 g/kg level, the commercial target needed to make brazzein at comparable costs to sucrose. This current level of expression appears to be well within cost-model targets for commercialization and a much lower cost option than that of the yeast host. Furthermore, stability tests on recombinant grain indicated it could be stored for years without loss of activity, making this a very practical production system. In addition, the maize-produced brazzein offers the option of milling to make sweet flour product for immediate use and without the need for purification. Therefore, stability testing and taste profiles could be done with purified material from either source and used interchangeably.

Brazzein was found to be stable for 6 h at 80 °C over a pH range from 3 to 8 when the purified product was used. Furthermore, the purified product was subjected to higher temperatures to simulate baking conditions. The results shown in Fig. 13.3 (D'Hoine, unpublished results) illustrate that even at temperatures of 100 °C (212 °F) over 1 h, there is still over 50 % initial amount of brazzein present.

While this provides hope for a highly stable product, this is no guarantee that the sweetness properties would be maintained as subtle changes in protein integrity may not show up using the biochemical assay. For this reason, prototype products were prepared using either purified brazzein or brazzein corn flour to replace sugar. An expert panel was used to assess sweetness in the final product to confirm that brazzein could withstand a variety of different processing conditions. Sweetness was observed in all cases (Table 13.2).

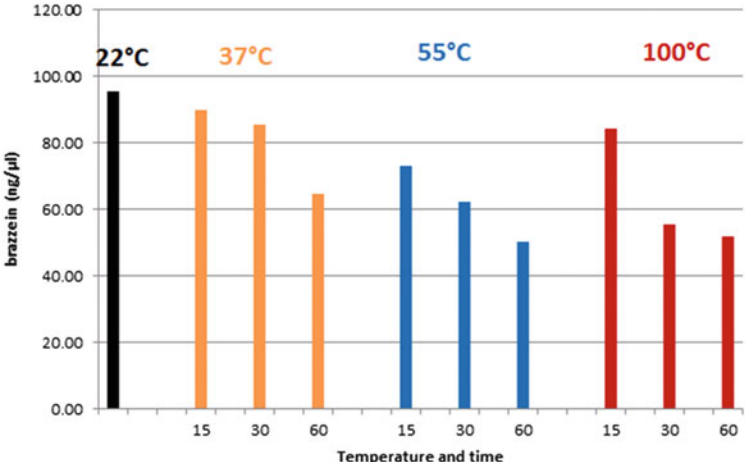

Fig. 13.3 Brazzein concentration in the purified form, baked at different temperatures and durations (From D'Hoine, unpublished results)

Table 13.2 Stability of brazzein in prototype products

Product	Form	Processing	Time	Sweet
Granola bars	Purified	400 °F	10 min	Yes
Yogurt	Purified	None	N/A	Yes
Pudding	Purified	Boiling	1 min	Yes
Corn puffs	Corn meal	70 °C	N/A	Yes
Muffins	Germ flour	325 °F	35 min	Yes
Corn bread	Meal/germ flour	400 °F	25 min	Yes

The prototype products were made using brazzein. Results indicated that sweetness was still present at the various processing conditions

13.4 Production

The commercial development of any product depends on the economic feasibility of its production. While expression in microbial systems was sufficient for pilot studies, large-scale production was determined to be impractical after making cost models take into account the cost of raw material and purification. As with microbial production, brazzein can also be extracted and purified from corn. Production in maize provides a number of advantages that allow it to be an economically viable option. First, at the expression level obtained in grain and using standard processing and protein purification techniques, the cost of the final product can be at or below the cost of sucrose on a sweetness equivalency. In addition, the generally regarded as safe (GRAS) status of corn provides the option to eliminate the purification step, a major financial hurdle. Therefore, in certain applications, sweet flour or a simple extract can be used rather than a highly purified product. This allows for a much-reduced cost. In addition, the regulatory pathway is streamlined as there is no traditional manufacturing step. In select applications, corn meal or corn flour can be used directly, a huge advantage over

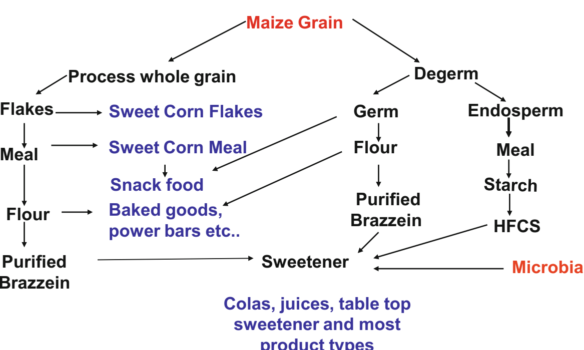

Fig. 13.4 Production options for brazzein. Starting with maize grain, a variety of options can be employed to use brazzein in food applications ranging from a highly purified sample similar to that when microbial production is used to whole grain flour that is sweet based on brazzein

other systems (Fig. 13.4). Based on cost models, it appears maize can meet or exceed cost targets in all methods of use. The current maize lines are expected to yield >1 kg of brazzein per ton seed processed when fully optimized. This is equivalent in sweetness to approximately 1,000 kg of sucrose. Given the preservation of other milled corn products such as oil, and starch, the value of this sweetness is approximately 100 % net added value. This system will yield a product at a competitive cost that is approximately equivalent to the cost of sucrose on a sweetener basis. Production lines have been developed that show the agronomic yield is not impacted with the brazzein protein that should enable a commercial product. Further development of production lines with even higher levels of brazzein is continuing that will further bring cost down.

When used directly as corn meal or flour, the low cost of materials can open up a number of new markets, specifically in whole grain snacks and cereals. A quick scan of ingredients among products on grocery market shelves shows that maize is currently being used in a myriad of products from beef stew to instant pudding.

13.5 Regulatory

13.5.1 Regulatory Clearance for a New, Consumed Product

In the USA, all new food substances must be approved for human consumption by the Food and Drug Administration (FDA). In addition, if this is to be made in a plant, the USDA also has jurisdiction. Therefore, a brief synopsis is given as to the regulatory hurdles. The FDA requires a series of stringent tests to ensure safety for a new commercial product. Since brazzein is a protein from an edible plant, it may overcome the first hurdle by virtue of its history of use in its native environment without adverse effects. This scenario is similar to thaumatin that was previously granted GRAS status. This potentially may reduce the effort required for regulatory

approval. Other studies required include acute toxicity, homology to toxins/allergens, and tests of protein degradation upon treatment with digestive enzymes.

The above studies represent the beginning of the process. Further regulatory requirements include a detailed physical/chemical analysis of the recombinant protein, a detailed description of the final product, and the detailed process for manufacturing. In the simplest case, corn flour already has GRAS status. Generally, if the molecule brazzein is granted GRAS status and maize has GRAS status, this should be a relatively straightforward procedure to obtain regulatory clearance. An aqueous extract of grain may also be considered GRAS; however, a purified protein will require more extensive data including details of the purification process.

Preliminary studies demonstrating no acute toxicity and no homology to toxin/allergens and the ability to hydrolyze with digestive enzymes have already been demonstrated (Howard, unpublished data). Longer-term toxicity studies and additional studies are also required. Typically, one can show toxicity at a given level for new molecules, but since the vast majority of proteins show little or no toxicity when given orally, rather than demonstrating toxicity, another approach is to demonstrate a no effect level. One important concern for proteins is the potential for allergenicity. There have been no reported cases of allergy to *P. brazzeana* to date, and a search against known allergen sequences has not revealed any reasons for concern. Nevertheless, this will have to be tested more fully before final approval is given.

13.5.2 Regulatory Clearance for Growing Transgenic Plants

In the USA, the US Department of Agriculture (USDA) regulates all transgenic crops initially. As 90 % of the corn grown in the USA is already transgenic, this does not raise any new concerns in general. The specifics of the transgenic product, however, must be addressed. As brazzein is a protein that readily degrades in the environment, and with FDA approval of safety on the molecule itself, this crop should be eligible for deregulation when the appropriate studies are completed. In the immediate case, growing of the crop must be done by containment meaning no other corn can be mixed during the growing, harvesting, processing, and shipping of the product. There are strict guidelines as to isolation distances and handling to ensure no intermixing of commodity crops. These requirements may be removed after deregulation, and the production should be similar to that of other identity preserved food crops.

13.5.3 Technical Hurdles

The largest technical hurdle to date has been to find a system that could produce the material at a cost competitive price. This appears to be solved by using the maize expression system to produce brazzein in many forms: as a highly purified product,

a less purified extract, or as corn flour/meal that can be used directly in food products. As this now appears that the cost hurdle can be overcome, the next major hurdle is regulatory approval. No insurmountable obstacles are foreseen in this area, but these will all take a large initial investment which does present a challenge to move this product to the marketplace. Another challenge will be to formulate brazzein with the vast array of different food products and to interface with current food processing techniques. Finally, there will be the task of scaling up to production levels. While there is no reason to believe this will be any more problematic than any other product, it still requires investment to finalize production lines and develop robust purification procedures and quality assurance guidelines to deliver a consistent product. With the right expertise, this should be relatively straightforward but will require additional time and money.

13.5.4 Nontechnical Hurdles

The conservative nature of food companies and lack of groundbreaking research presents a dilemma for new food products. The fact that brazzein has not yet undergone FDA approval creates a reluctance to even test the material by food companies. This puts brazzein in the infamous Catch 22 situation. Without regulatory approval, no one wants to develop products. Without developing test products, brazzein cannot get FDA approval. This is currently the largest hurdle to overcome. There needs to be a willingness to make a large investment to scale up production and obtain regulatory approval without the assurance that there will be a market. While this process is not new, it is a formidable barrier to entry, especially for food companies.

Another unknown is how the product will be labeled. Clearly, brazzein is a natural protein. However, will it still be labeled as a natural product if made in a recombinant host? There are other natural products made in fermentation using engineered corn, and as purified products, they bear the "natural" label. This situation appears to fit this model. A bigger question may be when using corn flour or corn meal. The dilemma may be since transgenic corn is in wide use today and does not have a label in the USA, can this product be considered natural if still in corn?

Currently, due to public opinion of genetically modified organism (GMO) products, there is a trend to avoid use of the term GMO whenever possible. This is much more of an issue in Europe than in the USA, but as food companies are international, this represents a challenge. For brazzein, however, a case can be made for a different approach. Rather than conceal the facts from the public with the suspicion that they cannot understand the process, let the consumers know upfront exactly what has been done and why. Then, they can choose whether the benefits of a low-calorie, protein sweetener are enough for them to purchase brazzein.

References

Gibbs BF, Alli I et al (1996) Sweet and taste-modifying proteins: a review. Nutr Res 16(9):1619–1630

Goyal S, Goyal R (2010) Stevia (Stevia rebaudiana) a bio-sweetener: a review. Int J Food Sci Nutr 61:1–10

Hellekant G, Danilova V (2005) Brazzein a small, sweet protein: discovery and physiological overview. Chem Senses 30(suppl 1):i88–i89

Lamphear BJ, Barker DK et al (2005) Expression of the sweet protein brazzein in maize for production of a new commercial sweetener. Plant Biotechnol J 3(1):103–114

Ming D, Hellekant G (1994) Brazzein, a new high-potency thermostable sweet protein from < i > Pentadiplandra brazzeana</i > B. FEBS Lett 355(1):106–108

Nabors LOB (2012) Alternative sweeteners. CRC, Boca Raton, FL

Priya K, Gupta VRM (2011) Natural sweeteners: a complete review. J Pharm Res 4(7):2034–2039

Part IV
Future Directions

Chapter 14
The Future of Plant-Produced Pharmaceuticals and Industrial Proteins

John Howard and Elizabeth E. Hood

14.1 Historical Perspective on Recombinant Protein Production

The initial host for producing recombinant proteins was *E. coli* because the technology was first developed using this bacterium. As the technology progressed, other organisms were used as alternative hosts, each with a potential advantage to produce certain types of proteins. The production of proteins is now a 50 billion dollar industry with most of the production being carried out in *E. coli*, yeast, or Chinese hamster ovary (CHO) cells (Andersen and Krummen 2002). Other systems continue to be developed to fill some of the unmet needs, including insect cultures and transgenic animals, because no one system appears to have solved all of the needs for every protein application.

Plants can also be used as a host, but this approach has greatly lagged behind the progress seen in other systems. This is in part because, as a higher organism, recombinant plant technology was more complicated than that required for microorganisms. Furthermore, the initial focus in plants was on crop improvement, and only a few groups experimented with plants as a host to produce recombinant proteins for industrial and pharmaceutical purposes.

Despite the fact that the earliest work of making industrial and pharmaceuticals was done in plants, the concept of using plants to make recombinant proteins for this purpose was met with much skepticism from the general scientific community. A wide range of technical questions need to be addressed such as: Could plants express microbial or animal proteins? Could plants perform posttranslational

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modifications on animal proteins? Could they form dimers, trimers, tetramers, etc. as in their native host? Would the proteins be stable if left on the crop until harvest? And could the proteins be readily purified from the complex plant matrix? Most of the recombinant protein production systems used today rely on microbial or cell culture systems where the culture medium and environment are highly controlled and can be manipulated at a moment's notice. Even though the origin of pharmaceutical and industrial products had its roots in plants, making these proteins in plants was clearly a paradigm shift from using dedicated growing vessels.

These technical questions were addressed by the scientific community with a host of excellent studies in a variety of systems providing insight to this general topic (Hood and Howard 2002). While there is still a lot to learn about plant-produced proteins, the technical question of can it be done has been answered, and today there is little doubt that plants can express a wide range of proteins.

The second question is, do any of these plant systems have the prerequisites for making commercially competitive proteins? Specifically, when is it practical to produce proteins in plants for commercial applications? Going beyond the basic theory, many groups have touted an array of potential advantages for a variety of different systems to produce recombinant proteins. As there are thousands of different plant systems that can be used, the question in front of us now is which, if any, of these are useful in a commercial sense? Some general guidelines have been proposed as to when certain plant systems may be preferred that may help at a very basic level (Howard and Hood 2005), but the technology continues to develop, and each protein has its own unique set of challenges. Nevertheless, we can now definitively answer the second question as to "can it be done?" since the first plant-produced recombinant proteins (β-glucuronidase and avidin) were commercialized 15 years ago (Hood et al. 1997; Witcher et al. 1998). This success addressed a host of practical questions as to functional equivalency, storage, and purification of the proteins. This commercialization event also highlighted some key advantages including using an animal-free source, reduced cost of raw material, and reduced cost of processing. Today there are more than 30 proteins (Table 14.1) that have been commercialized. These have been for industrial and health-related applications, and the first plant-produced recombinant therapeutic protein was announced last year (http://www.protalix.com/procellex-platform/overview-procellex-platform.asp).

Despite the commercialization of these products, this is an extremely modest amount of activity compared to the industry at large. The success in plants represents what is better labeled a niche market rather than mainstream. So now the question is, what is holding up plants to becoming one of the mainstream systems for protein production? The theoretical advantages touted in many review articles are convincing, and the basic science and commercialization questions have been addressed, yet the industry has moved very slowly. What follows is an opinion of why the industry has moved slowly, what needs to happen for plants to play a major role, and which applications are most likely to be affected and when.

Table 14.1 Commercialized plant-produced recombinant proteins

Protein	Company	Host
ß-Glucuronidase	ProdiGene	Maize
Avidin	ProdiGene	Maize
Exocellulase CBHI	IE/ABI	Maize
Endocellulase E1	IE/ABI	Maize
Trypsin	ABI/ProdiGene	Maize
Aprotinin	LSBC	Tobacco
Lysozyme	Ventria	Rice
Lactoferrin	Ventria	Rice
Serum albumin	Ventria	Rice
Transferrin	Ventria	Rice
Leukemia inhibitory factor	Ventria	Rice
Glucocerebrosidase	Protalix	Carrot cells
Over 20 more proteins[a]—examples include:	ORF Genetics	Barley
Activins A and B	ORF Genetics	Barley
Interferon-gamma	ORF Genetics	Barley
Interleukins 2,3,4, and 6	ORF Genetics	Barley
Stem cell factor	ORF Genetics	Barley
Tumor necrosis factor-alpha	ORF Genetics	Barley

A partial list of recombinant proteins that have been commercialized. The company responsible for development is listed along with the host tissue. *IE* Infinite Enzymes, *ABI* Applied Biotechnology Institute, *LSBC* Large Scale Biology Company

[a]For the full list of products from ORF Genetics, see http://2ww.orfgenetics.com/ISOkine/ProductList/

14.2 Why Has It Taken So Long to Develop the Plant Platform?

If plants are a better platform for expressing proteins, then why has it taken so long for this to occur while other systems dominate the industry? The first reason is that basic technology funding for plants is only a fraction of the funding that is available to microbial and human health research. Therefore, it took longer to develop the basic scientific principles needed for recombinant technology in plants. This is only a partial answer of course and was exacerbated by several other factors. As plants are higher eukaryotes, they are much more complicated than microorganisms, which included a basic and practical understanding of aspects of protein accumulation as it differs from microorganisms such as regulation of expression in different tissues and intracellular locations and developmental stage of the plant. Therefore, more funding was needed to discern even the basic biology. Progress was further complicated by the fact that plants represent a kingdom with thousands of viable options for production, each with its own peculiarities. Even if we focus on cultivated plants, we still have hundreds of choices. Finally, the vast majority of the funding that went to plants for recombinant DNA technology was focused on crop improvement. While crop improvement was needed and some of the technology did cross over, only a few small groups with limited budgets initially tried to make the case for this

work, and they did not have the backing of government institutions or private industry similar to that offered to non-plant protein production systems.

Funding to create plant production technology was not the only factor slowing technology development. Fear of the unknown and resistance to change usually play a role in the acceptance of any new technology, and this is no exception. Major corporations had established research and production platforms for microbial production. No enthusiasm existed to fund work that would replace billions of dollars of investment and dedicated production facilities. The fear of upsetting the status quo could be felt by anyone trying to suggest that another way to accomplish the goal of protein production may be available.

Finally, fear in the general population has also contributed to the delay in acceptance. The debate over genetic engineering (GE) has spilled over from the use of recombinant DNA in foods to the use of plants to produce pharmaceutical and industrial proteins in plants as well. The fact that bacteria and yeast are food organisms used extensively as host for genetically engineered proteins does not seem to penetrate into the public's debate. The fact that many of the proteins that are proposed to be made in plants are already in the food chain also does not seem to matter. In general, safety is not the focus of these debates at all but rather emotional issues. Regulatory guidelines have been instituted for decades in order to produce pharmaceutical and industrial proteins from native or recombinant hosts. This also includes keeping these segregated from the food supply no matter if this is bacteria, yeast, eggs, or plants. In addition, new sets of guidelines (enforced by the USDA in the USA) have been introduced to specifically address the production of industrial and pharmaceutical recombinant proteins in plants. While fear and bias can eventually be replaced with logic and a risk-benefit analysis, the heightened sensitivity of genetically engineered crops has delayed reason and at times put safety concerns in the background.

14.3 What Is Needed to Use Plants as a Major Technology for Protein Production?

Several factors need to be addressed for plant production systems to become a mainstream technology in the production of recombinant proteins. These factors include overcoming both technical and nontechnical barriers. On the nontechnical front, there must be a willingness to fund the work to create the products, and the public must accept the products when produced. While this seems obvious and in many cases not contentious, the fact that these are GE products has raised concerns. While the GE debate is most active as it relates to food safety and there are no documented scientific reports of anyone getting sick from a GE food, this has not been sufficient to dissuade the public concerns. Although there is still a strong opposition based on perceived safety issues, GE food has become part of the mainstream.

Like most any new technology, theoretical risk (fear) usually wins over theoretical benefits (promises). When the promises are transformed into real benefits, then

the risks are more keenly evaluated. This is true for the GE food debate and likely to be true with any opposition that may be encountered to plant-produced proteins. One advantage for plant-produced proteins that was not as clear for food applications is that the public has a clear choice to use the product or not and can evaluate their risk and their benefit directly. Therefore, it is hoped, but not guaranteed, that plant-produced proteins will be accepted as they have from the other hosts of industrial and pharmaceutical products.

The trigger for accepting plant-produced proteins has begun by providing an animal-free source of proteins as discussed below. In this case, the perceived risk of not using plants to produce the proteins is greater than the perceived risks of using them. In other words, the fear of GE plants apparently is not as bad as the real risk of having a viral or prion contamination in a therapeutic product. This formula of establishing direct benefits to the consumer will be the key to acceptance of this technology as it relates to consumers. The use of plant-produced proteins to make animal-free products illustrates an important point, the technology will not gain acceptance if it is at par with existing systems. The new technology must have some compelling advantage to overcome the status quo.

One compelling reason is if plants can provide a dramatic cost advantage. The cost of most proteins is inversely proportional to the concentration in the host tissue. In the past, protein levels produced in plants were not high enough to translate to a dramatic cost advantage, but this began to change in recent years. Levels of recombinant proteins in some grains and tobacco leaves for selected proteins are well within the realm of other systems as it relates to cost. Still, this only works today when coupled with other advantages that can offset the cost of changing systems. To gain long-term acceptance, several technology improvements can help decrease the overall cost and are discussed below.

14.4 Technology Improvements Needed for Industrial Applications

14.4.1 Accumulation of Recombinant Proteins in Host Tissue

This is the most crucial factor in reducing cost. High expression results not only in a lower cost of raw material but a lower cost in transportation, storage, processing, and purification. In microbial systems, more than 70 % of the energy inputs can be converted to the final product. In plants when the recombinant protein reaches at least 1 % of the weight of the tissue, it is considered very good expression but obviously is not nearly 70 % of the inputs. Clearly there is still much more room for improvement in plants to reach higher levels of accumulation for any protein. Some general rules for the best ways to express specific types of proteins in plants are needed.

14.4.2 Product Integrity

Proteins made in plants are usually very similar if not identical to those made in other systems. There are examples, however, where plants do not possess the same type of machinery as animals and modifications can be made. One example of this is when plants do not have the required enzymes for posttranslation modifications. The case most often cited is that of the differences in glycosylation between plants and animals. While both plants and animals share the basic backbone in protein glycosylation patterns, there are subtle differences in linkages and sugar composition. Theoretical debates over the potential for allergenicity have been waged over these differences. However, one can also make a theoretical argument that as we eat plant proteins routinely, this difference does not necessarily lead to allergenicity. Testing a number of proteins over the years will demonstrate how important this difference in glycosylation is in practice, but the cases studied to date have not shown any indication that these differences lead to allergenicity.

Another example of altered glycosylation patterns is the addition of sialic acid on some select animal proteins. While there is no evidence for the presence or absence of this sugar to alter activity or lead to allergenicity, sialic acid can prolong the retention time of proteins in the blood (Morell et al. 1971). The differences in glycosylation have led to modifications of the plant's machinery to make glycosylation similar to that present in animals (Jez et al. 2013). These changes are not always essential, but having them included in future protein design eliminates concerns and would be a valuable contribution in the long term.

Glycosylation is not the only case when plants may require other posttranslational enzymes. As an example, in addition to the gene-encoding collagen, a second gene was introduced that performed the required hydroxylation to generate the preferred form of the protein (Xu et al. 2011). This example along with the glycosylation enzymes may represent an opportunity in the future where certain plants may be engineered to possess specific posttranslational enzymes that can be used for pharmaceutical purposes.

14.4.3 Tissue Processing/Purification

The cost of purification usually represents greater than 80 % of the overall cost of a typical pharmaceutical protein. Much of the protein purification technology can be adapted from that used in other non-plant systems, but there are some unique aspects that need to be addressed for plant systems. In the case of the grains, many options for established processing technologies are available that can be adapted. This is in contrast to vegetative tissue such as tobacco leaves where much of the technology had to be developed for this specific purpose. It will be important to understand what can be improved upon in downstream engineering or in modification of the starting genetic material to eliminate or reduce toxic compounds or agents that may interfere in purification.

Finally, the possibility of by-products or coproducts of plant tissue can help reduce the overall costs. If only one part of the host tissue expresses the protein of interest, the rest of the plant may be available for other products. One example of this is illustrated when using corn germ to produce recombinant proteins leaving the endosperm for other uses such as the production of ethanol (Howard and Hood 2007). While this is not a critical factor for higher priced pharmaceuticals, coproducts can become critical for some industrial proteins. To be able to practice this in full will require the infrastructure for an integrated production facility and the regulatory approvals to allow uses of the other parts of the plant.

14.4.4 Development Time

The time required to transform plants is measured in months as opposed to hours or days as in the case of most microorganisms. It is not uncommon for many plants to have a year's delay before the first prototype product can be evaluated. While in the long run this is not a major deterrent, there is no guarantee that the protein was made correctly, and waiting a year to make the second version is a major drawback. The time it takes to evaluate these new constructs needs to be significantly reduced. In the case of the transient expression system in tobacco leaves, the development time has been reduced to weeks (Fischer et al. 1999; Scholthof et al. 1996). While still much longer than that of microbes, it is practical to work around many of the limitations to make this competitive with overall developmental times in microbes. This situation however is not the case for stable transformation systems, especially in the grains. Significant resources will eventually be required to address this problem.

14.4.5 Product and Crop Containment

Regulatory guidelines are in place to assure product integrity and containment when expressing proteins in any host. Regulation has been used successfully for bacteria, yeast, eggs, and a variety of other microorganisms. While plants represent a new host, the same regulatory guidelines for production, storage, transportation, and containment apply. There are some unique aspects to plants such as growing the crop outdoors rather than in a contained building, which are significantly different. For this reason, USDA has also imposed guidelines for containment to ensure no inadvertent escape into the environment, or mixing into the food or feed chain.

In addition to containment of the organisms, several new genetic strains of microorganisms have been developed that have characteristics that benefit the accumulation of recombinant proteins such as enabling higher levels of accumulation. The concept of having genetic strains that are better suited for containment and expression in microorganisms has not crossed over to plants. This is one area that would greatly benefit the commercial application of the plant system and at the

Table 14.2 Key attributes of future germplasm designed for plant-produced pharmaceutical and industrial proteins

Trait	Description	Benefit
High protein lines	Diverting more of the plant's resources to making proteins rather than oils or carbohydrates in the desired tissue	Greater recombinant protein accumulation resulting in a lower cost of production
Humanized glycosylation	Lines engineered that will allow glycosylation patterns similar to those in humans such as the addition of sialic acid	Necessary for some pharmaceutical proteins and reduced concerns for allergenicity
Specific selectable markers	Herbicide resistance markers that are only used for this purpose to easily distinguish them from transgenic crops used for food or feed	Easier to eliminate in fields and create genetic safeguards for escapes
Colored seed coat	Having a different colored seed coat to easily distinguish it from food or feed seed	Create additional safeguards to improve public confidence
Male sterility	Create genetic systems to replace manual sterilization	Additional safeguards for inadvertent pollination can reduce isolation requirements resulting in lower costs
Germination control	Allow germination only to occur when responding to a specific chemical	Prevent unwanted seed from germinating, creating greater public confidence

same time provide additional safeguards for containment. The proposal is that specific plant germplasm would be developed that is optimized for plant-produced pharmaceutical and industrial proteins. Some of the key attributes that may be included are shown in Table 14.2. It is important to recognize that none of the items listed are essential. Conditions for containment are met now mostly by isolation requirements, and protein accumulation is adequate for many products. Nevertheless, the changes below represent the future enablement of genetic safeguards for containment that would be much less cumbersome and costly than the isolation methods used today. The increase in protein expression would allow more proteins to be commercialized at a reduced cost, benefitting the industry and the public. It is recognized that it will require significant resources over a long period of time to incorporate many of these features, but in the long term this will reduce the overall cost of production and give the public greater confidence.

14.5 What Applications Are Most Likely to Use This Technology and When?

Every protein production platform has a list of advantages as to why it is better than other platforms using competitive technologies. From a commercial perspective, all of the arguments can all be broken down to cost. In a standard definition of the unit cost of

production, this entails the generation of raw material, storage, transportation processing, and purification. Other factors also can also contribute to the overall cost as well, such as the cost of research needed to make the product, the cost of meeting regulatory requirements, the product formulations, the capital cost of building new production facilities, the cost of changing over to a new system, and even the cost of public education if needed to gain acceptance of new products. What this means is that in order to launch a new platform technology, it is not enough to compete on the traditional unit cost of production alone. Additional advantages must overcome the hidden cost of these barriers particularly when replacement of existing infrastructure is required. The acceptance of this new technology relies on this broader definition of cost that is not always well defined in economic terms but clearly present in making production decisions. Therefore, when we discuss the applications below, it is always in the context of this broader overall cost.

14.5.1 Fine Chemicals

The first commercial plant-produced products could be considered to be in the fine chemicals market, consisting of specialized proteins used for research, diagnostics, or other specialized uses. In general, the proteins are used to make other products. The advantage here is that if the proteins are functionally equivalent, they can be substituted directly for the existing product without making any other infrastructure changes and require little regulatory oversight. Easy introduction into the market place can occur and does not involve the general public.

While the first plant-produced products were functionally equivalent to native proteins and the unit cost of production was lower than the native protein, they did not offer any other clear advantage. A convincing reason as to why someone should stop using what they were accustomed to and switch to a new product was not obvious. This type of product with a relatively low volume, small market value, and large expense of research and development (R&D) cannot be justified to make new versions of the products. This was definitely true 15 years ago and, although the technology has improved dramatically, is still the case today. Even though the production of these proteins may have a clear demonstrated lower unit cost, the cost of developing the products must come down dramatically for this to be a significant contributor in the market. When the infrastructure for plant-produced proteins becomes mature, replacement products may be possible. This application remains a niche market, however, and will not bring the technology into the mainstream.

14.5.2 Animal-Free Source of Proteins for Health Care (Nontherapeutics)

The first significant commercial success in using plant-produced recombinant proteins is just now being realized in the production of animal-free proteins. The

technology has found a home due to the regulatory impetus in Europe and in the USA to require pharmaceutical manufacturing to use animal-free systems whenever feasible. Many of these products recently introduced are used in the manufacturing of pharmaceuticals with animal cell culture systems that account for a large portion of the current manufacturing of therapeutics. While cell culture facilities are developed in a very clean environment, they often rely on animal proteins such as growth factors and processing enzymes to produce the final therapeutic product. These required proteins are not easily manufactured in microbes because of post-translational requirements. Obtaining them from their native source is not always practical as is the case for some human proteins or desirable as in the case of some animal proteins that bring the threat of pathogen contamination. In general, these plant-made versions offer a good alternative, even though they are priced at a premium over the native proteins. This is a clear instance when unit cost of production is not the driving factor.

The trend toward more protein therapeutics and guidelines to use animal-free sources would suggest that this market will continue to increase in the near future. As many of these proteins require posttranslational modifications, microbial production is not suitable, providing the opening for plant-produced products. This trend, however, is not limited to the use in cell culture systems as illustrated by the case for trypsin (Chap. 4). In cases where microbial production is preferred, there still may be a requirement for posttranslational processing using plant-produced proteins.

14.5.3 Therapeutics

Therapeutics represents the largest market by far for recombinant protein products. This is also the hardest market to enter due to the expense and time-encumbered regulatory studies. It is difficult to justify the added expenses of repeating regulatory studies and replacing expensive manufacturing facilities for the same protein product that is simply a little less expensive to produce. The hurdle that must be overcome is that the product must have a large market to justify the expense and the cost reduction must be dramatic.

Another option is to produce new products rather than the same product in a different system. In this case making the new product in the plant system at the start of the regulatory process will eliminate the hurdle of duplicating regulatory studies. The catch is that it is difficult to have a company try an untested production system for their blockbuster product. This catch 22, however, may be broken at least in part with the introduction of taliglucerase by Protalix/Pfizer in the treatment of Gaucher's disease. This represents the first plant-produced recombinant therapeutic to reach the market. While not a blockbuster drug, taliglucerase does represent a case where a regulatory path can be outlined in detail and should help provide confidence to large companies to test other plant-produced products.

A slow acceptance of this technology will continue for therapeutics until a blockbuster product is made. At that time, the plant platform will be considered mainstream and at par with other platforms where production decisions will be made as to which system can produce the product at the lowest unit cost. In addition, with mainstream acceptance, the production of generics should be more attractive assuming the unit cost is lower in the plant system. When this occurs, the market for an animal-free source of proteins used in for the manufacturing of therapeutics in cell culture production systems may start to decline albeit very slowly.

14.5.4 Vaccines

One area that has received much attention is the use of plants to produce vaccines. While no products are currently on the market, several clinical trials have been conducted, and many of the practical problems appear to have been resolved as products move ahead in the pipeline toward commercialization. Two very different approaches utilize two different types of platforms.

First, in the case of pathogens that mutate quickly, such as many of the flu viruses, having a eukaryotic production system that can be employed rapidly to keep pace with the mutating virus is a clear advantage. The transient expression of vaccines in tobacco leaves can meet this need, and it seems likely that the first products are underway as they move through the required regulatory studies. A few examples are illustrated in this book, but more are likely to emerge as soon as the first of these is commercialized.

The second application is the use of plants to make oral vaccines. While conceptually this sounds like a clear advantage, difficulties in expressing the antigens at high concentrations have limited progress. However, progress has been made on this front, and this technical barrier now seems to be resolved. The acceptance of this type of product is more complicated than that of parenteral vaccines. The oral route of administration requires more studies to assure that this method will provide adequate protection since the final product is not the same as that made from other sources. In addition, many vaccines are co-administered in one injection; therefore, replacing one of the injected vaccines does not eliminate the need for the injection. While these problems can be resolved in the long term, in the short term they are barriers. The approach to using a plant-made vaccine as a booster may be the best way for this new type of product to be introduced and a way to monitor the effects of the immune system in a large population with little risk.

An oral vaccine made from plants also illustrates an important point in commercialization. The new product has benefits well beyond the simple economic cost model for making a protein at a lower cost. The plant platform impacts the way the end user receives the product, and it disrupts current dogma. Not only can the product itself be produced less expensively but also the downstream needs of administration are also greatly reduced adding enormous value. While this example illustrates the greatest potential benefit, it also has the greatest barriers to overcome.

14.5.5 Food and Feed Additives

The public debate over GE foods has clearly disincentivized any work in this area. Ironically, however, from a safety perspective, the choice of using an animal-free source of proteins for processing food (particularly if it is made in a food crop with generally recognized as safe (GRAS) status) is much less risky than using the same protein made from animals that may carry contaminating pathogens. Once emotional issues have receded, logic will hopefully win in the longer term. The idea of making functional foods is not new, and having these plant-produced proteins replace animal proteins should be a lower risk alternative that the public can embrace once they get past the emotional reactions to GE foods. Once this occurs, food processing is likely to become a major application for plant-produced proteins.

In addition to reducing risk, a much greater incentive to use this approach is the potential to add proteins to food at a cost that would otherwise be prohibitive. The example of brazzein (Chap. 13) illustrates the case where the protein can add value without placing a large burden on cost since purification of the protein is not necessary. Another example is the use of feed or food supplements to aid in digestion. Protease and cellulase from native sources are already used in this manner, and these proteins have been expressed in plants as well (Chaps. 4 and 12). What is missing is the will to test these plant-produced enzymes and demonstrate that these will work in the specific applications. These proteins have the potential to turn the public's attention away from the fear of GE food and put it on the function and safety of food products. In the future, labeling of GE food may even be a selling point to highlight that unlike other forms of adding new proteins to the crop that do not involve genetic engineering, these products have been safety tested and contain functional benefits that the consumer can appreciate.

14.5.6 Industrial Proteins

One advantage that has been touted consistently for plant-produced proteins is that extremely large volumes can be made at a very low cost. One example that illustrates this unmet need is the requirement to produce enzymes for the conversion to bioethanol from cellulose. Chapter 12 reviews this in more detail, and it is hard to imagine how other protein production systems can compete with the volume and cost parameters required for this type of project. The first technical problem to achieve very low cost is to have very high expression levels. The next problem is to obtain activity of the enzymes in crude extracts because purification of the enzymes would be cost prohibitive. In examples where only one enzyme is needed, this example sets a good precedent for volume and cost. This specific case will also require the action of other enzymes for bioconversion of cellulose that must be made in plants or microbes to have a fully functional application. The plant system has this potential, but it also illustrates the contrast with the fungal secreted

enzymes. In plants there is a systematic and concrete approach of engineering one enzyme at a time which is in contrast to using fungal isolates that may secrete 20 enzymes and have been selected for the ability to deconstruct cellulose. Each production system has its advantages, and choice of a system depends ultimately on its final application.

14.6 Conclusions

The commercialization of plant-produced proteins has progressed slowly over the past 15 years since the first introduction of a commercial product demonstrating feasibility. Many factors have contributed to this slow progress, but in brief, the technology was not robust and predictable in the early stages to compete strictly on a cost basis with other existing platforms, and there was little motivation to fund technology improvements to a system that was considered a threat to existing platforms. In the last several years, however, the advantages of plant production systems beyond the unit costs are enabling the acceptance of the technology. The clear front-runner is the move into an animal-free source of proteins for cell cultures. This may soon be followed by an animal-free source of therapeutics, a rapid system for the production of parenteral vaccines, orally delivered vaccines, and industrial enzymes that can only be produced on the scale that a plant system can provide. The advantages of plant-produced proteins beyond the unit cost are the key to the initial commercialization. In the longer term as the technology becomes more engrained into the industry, this approach can be used for a variety of other proteins where plants can compete on unit cost as well.

References

Andersen DC, Krummen L (2002) Recombinant protein expression for therapeutic applications. Curr Opin Biotechnol 13:117–123

Fischer R, Vaquero-Martin C, Sack M, Drossard J, Emans N, Commandeur U (1999) Towards molecular farming in the future: transient protein expression in plants. Biotechnol Appl Biochem 30:113–116

Hood EE, Howard JA (2002) Plants as factories for protein production. Kluwer Academic, Dordrecht

Hood EE, Witcher DR, Maddock S, Meyer T, Baszczynski C, Bailey M, Flynn P, Register J, Marshall L, Bond D, Kulisek E, Kusnadi A, Evangelista R, Nikolov Z, Wooge C, Mehigh RJ, Herman R, Kappel WK, Ritland D, Li C-P, Howard JA (1997) Commercial production of avidin from transgenic maize: characterization of transformant, production, processing, extraction and purification. Mol Breed 3:291–306

Howard J, Hood E (2005) Bioindustrial and biopharmaceutical products produced in plants. Adv Agron 85:91–124

Howard JA, Hood EE (2007) Methods for growing nonfood products in transgenic plants. Crop Sci 47:1255–1262

Jez J, Castilho A, Grass J, Vorauer-Uhl K, Sterovsky T, Altmann F, Steinkellner H (2013) Expression of functionally active sialylated human erythropoietin in plants. Biotechnol J 8:371–382

Morell AG, Gregoriadis G, Scheinberg IH, Hickman J, Ashwell G (1971) The role of sialic acid in determining the survival of glycoproteins in the circulation. J Biol Chem 246:1461–1467

Scholthof HB, Scholthof K-BG, Jackson AO (1996) Plant virus gene vectors for transient expression of foreign proteins in plants. Annu Rev Phytopathol 34:299–323

Witcher DR, Hood EE, Peterson D, Bailey M, Bond D, Kusnadi A, Evangelista R, Nikolov Z, Wooge C, Mehigh R, Kappe W, Register J, Howard JA (1998) Commercial production of beta-glucuronidase (GUS): a model system for the production of proteins in plants. Mol Breed 4:301–312

Xu X, Gan Q, Clough RC, Pappu KM, Howard JA, Baez JA, Wang K (2011) Hydroxylation of recombinant human collagen type I alpha 1 in transgenic maize co-expressed with a recombinant human prolyl 4-hydroxylase. BMC Biotechnol 11:69

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