Plant-produced candidate countermeasures against emerging and reemerging infections and bioterror agents

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Summary
Despite progress in the prevention and treatment of infectious diseases, they continue to present a major threat to public health. The frequency of emerging and reemerging infections and the risk of bioterrorism warrant significant efforts towards the development of prophylactic and therapeutic countermeasures. Vaccines are the mainstay of infectious disease prophylaxis. Traditional vaccines, however, are failing to satisfy the global demand because of limited scalability of production systems, long production timelines and product safety concerns. Subunit vaccines are a highly promising alternative to traditional vaccines. Subunit vaccines, as well as monoclonal antibodies and other therapeutic proteins, can be produced in heterologous expression systems based on bacteria, yeast, insect cells or mammalian cells, in shorter times and at higher quantities, and are efficacious and safe. However, current recombinant systems have certain limitations associated with production capacity and cost. Plants are emerging as a promising platform for recombinant protein production due to time and cost efficiency, scalability, lack of harboured mammalian pathogens and possession of the machinery for eukaryotic post-translational protein modification. So far, a variety of subunit vaccines, monoclonal antibodies and therapeutic proteins (antivirals) have been produced in plants as candidate countermeasures against emerging, reemerging and bioterrorism-related infections. Many of these have been extensively evaluated in animal models and some have shown safety and immunogenicity in clinical trials. Here, we overview ongoing efforts to producing such plant-based countermeasures.

Introduction
Despite major progress in the prevention and treatment of infectious diseases, particularly the development of antibiotics and vaccines, they remain the second leading cause of death worldwide (http://www.niaid.nih.gov/about/WhoWeAre/Documents/niastrategicplan2008.pdf). According to the World Health Organization (WHO), 11.5% of approximately 56 million deaths in 2012 were caused by infectious diseases (http://www.who.int/healthinfo/global_burden_disease/estimates/en/index1.html). Furthermore, approximately 50% of all deaths among children under 5 years of age in 2013 were due to infectious diseases (http://www.who.int/gho/child_health/mortality/causes/en/).

Emergence of new infections and reemergence of known infections are the main obstacles on the way towards combating infectious diseases. Over 60% of emerging infections are caused by zoonotic pathogens, and approximately 72% of those are caused by pathogens derived from wildlife (Jones et al., 2008). Contributing factors are often associated with human activity, resulting in increased human exposure to animal pathogens (http://www.niaid.nih.gov/topics/emerging/pages/introduction.aspx). For example, changes in human demographics and behaviour caused outbreaks of Chikungunya virus infection in Tanzania in 1952, poliomyelitis in India in 2003 and severe acute respiratory syndrome (SARS) in China in 2002, while changes in the land use and agricultural industry caused outbreaks of plague in the United States in 1970, European tick-borne encephalitis in Norway and Far Eastern tick-borne encephalitis in Japan in 1993, Nipah virus infection in Malaysia in 1998 and Rift Valley fever in Egypt in 1977 (Jones et al., 2008). Furthermore, the excessive use of antimicrobial agents contributes to the development of drug-resistant strains of pathogens such as Mycobacterium tuberculosis and Plasmodium falciparum. In addition to human activity, natural genetic variation and recombination and adaptation of known pathogens, such as influenza viruses, result in the appearance of new strains with epidemic and pandemic potential.

Besides those transmitted naturally, infectious diseases can also emerge via intentional release or dissemination of unmodified or engineered forms of life-threatening pathogens or biological toxins, used as agents of biowarfare or bioterrorism (http://www.niaid.nih.gov/topics/emerging; Balali-Mood et al., 2013). The use of microbes and their products as bioweapons has a long history (Hilleman, 2002). Poisoning and faecal contamination of arrows, spears and punji sticks were practised in ancient times. Contamination of water and food supplies, the projection of infected animal or human cadavers into enemy camps and release of infected rats were also employed in later centuries. By the beginning of the 20th century, biological warfare had acquired a
which was based on formaldehyde-killed whole-cell BioSolutions, 2012). The licensed smallpox vaccine is generally well tolerated; pneumonia form of the disease (Hassani et al., 2004; Meyer, 1970). The licensed smallpox vaccine is generally well tolerated; however, it may cause serious side effects in approximately 10% of vaccinated individuals and also did not protect against the pneumonic form of the disease (Hassani et al., 2001), the risk of bioterrorism has remained high, promoting significant efforts towards the development of diagnostic, prophylactic and therapeutic measures for biodefence (http://www.niaid.nih.gov/about/whoWeAre/Documents/niaidstrategicplan2008.pdf).

Vaccines are the mainstay of infectious disease prophylaxis, and a few have been approved in the United States for prevention of emerging, reemerging and bioterror infections. Target populations for these vaccines vary, from general populations in case of seasonal influenza to populations living in or travelling to endemic areas in case of yellow fever to individuals at high risk of exposure such as the military personnel. The majority of approved vaccines against infectious diseases are based on live attenuated or killed whole pathogens. Despite demonstrated protective efficacy, most of them have limitations. Safety is the major issue with these vaccines. For example, BioThrax®, the vaccine against anthrax, is based on protective antigen (PA) of B. anthracis, but may also contain other components of the bacterium, such as lethal factor (LF) and oedema factor (EF). Therefore, the composition of BioThrax® is not fully defined and varies from lot to lot. Because of the presence of LF and EF, the vaccine may cause severe allergic reactions including anaphylaxis (Emergent BioSolutions, 2012). In addition, to induce protective immunity, BioThrax® should be administered in as many as five doses over a period of 18 months and at 1-year intervals thereafter (Emergent BioSolutions, 2012).

Similarly, vaccine against plague, currently discontinued, which was based on formaldehyde-killed whole-cell Yersinia pestis, caused serious side effects in approximately 10% of vaccinated individuals and also did not protect against the pneumonic form of the disease (Hassani et al., 2004; Meyer, 1970). The licensed smallpox vaccine is generally well tolerated; however, it may cause serious side effects in individuals with immune deficiencies or skin disorders (www.niaid.nih.gov/topics/smallpox).

Another licensed vaccine, YF-Vax®, against yellow fever, in rare cases, causes encephalitis and other neurologic syndromes. Moreover, the vaccine can cause a severe visceralotropic disease similar to wild-type YF, resulting from active YF virus replication in a genetically predisposed host and direct viral injury to vital organs (Lindsey et al., 2009).

In addition to safety concerns, some traditional vaccines need to be frequently redesigned to address genetic variations in target pathogen strains. For example, circulating influenza viruses undergo constant antigenic drift and occasional antigenic shift (http://www.cdc.gov/flu/about/viruses/change.htm), and mutations may occur in egg-adapted virus strains, resulting in reduced vaccine efficacy (Skowronski et al., 2014). In the case of seasonal trivalent influenza vaccines, the preparation of the first vaccine lot for a new influenza season takes 4–5 months after the date of vaccine strain selection (Matthews, 2006).

Overall, the recent emergence of new infections, outbreaks of existing infections and the threat of bioterror attacks to the well-being of global populations underscore the urgent need for robust and highly scalable alternative manufacturing systems, independent of the propagation of live bacteria and viruses, virus strain adaptation and traditional matrices such as eggs. These systems are expected to be capable of producing safe, effective and affordable vaccines that can be administered in few doses and produced in high quantities within a short time frame, providing a rapid response.

Subunit vaccines, based on a recombinant pathogen’s proteins or peptides and produced in heterologous systems, are a highly promising alternative to whole pathogen- or whole live-engineered strain-based vaccines. So far, five subunit vaccines have been licensed, four of which are based on highly purified virus-like particles (VLPs) [Engerix® (GlaxoSmithKline, 2013) and Recombivax HB® (Merck, 2014) against hepatitis B virus and Cervarix® (GlaxoSmithKline, 2015) and Gardasil® (Merck, 2015) against human papillomavirus]. Besides subunit vaccines, human monoclonal antibodies, antiviral agents and immune modulators are also promising prophylaxis and treatment measures to control emerging and bioterror-related infections (Bishop, 2015; Schleich et al., 2011; Shurtleff, 2004; Zhu et al., 2006). These proteins can prevent or reduce the severity of diseases by variety of mechanisms. For example, monoclonal antibodies can neutralize pathogens growth, limit their spread from infected to adjacent cells, or inhibit the biological activity of toxins.

Depending on the size, structure and post-translational decorations of the target recombinant protein, as well as cost considerations, a choice of heterologous expression system can be made: bacteria, yeast, insect cell or mammalian cell-based (Celik and Calık, 2012; Chen, 2012; Drugmand et al., 2012; Zhu, 2012). These production platforms are scalable and flexible; however, each of them has certain limitations associated with product efficacy, safety, response time, scalability and cost.

Plants have long been proposed as an attractive platform for the production of recombinant proteins for human health, because of perceived safety advantages, as they do not harbour mammalian pathogens, and cost and scalability advantages, as stainless steel fermenters are not required. Results of studies conducted during the last two decades suggest that plants are cost-effective, highly scalable and safe platforms for the production of subunit vaccines, monoclonal antibodies and therapeutic proteins (Basaran and Rodríguez-Cerezo, 2008; Stoger et al., 2014; Tiwari et al., 2009, Twyman et al., 2012; Xu et al., 2012). In addition, plant cells are capable of performing eukaryotic post-translational modifications (PTM) of target proteins, including N-linked glycosylation, which are substantially similar to those found in mammalian cells (Gomord and Faye, 2004).

However, while the core glycan structure is shared by mammalian and plant N-linked glycans, terminal residues differ.
Plant-derived recombinant proteins contain terminal bisecting β1,2-xylose and core α1,3-fucose, but no β1,4-galactose and sialic acid residues (Bosch et al., 2013; Gomord et al., 2010; Webster and Thomas, 2012). Plants and mammals also differ in the structure of O-linked glycans that, in contrast to N-linked glycans, are synthesized by a stepwise transfer of monosaccharides in the endoplasmic reticulum (ER) and the Golgi complex. While mammalian cells synthesize mucin-type O-linked glycans, plant cells produce extensin- and arabino-galactan-type O-linked glycans (Gomord et al., 2010).

Several studies have been conducted to examine potential negative effects of plant-specific glycans on the safety and efficacy of plant-produced subunit vaccines and therapeutic proteins. For example, glucocerebrosidase produced in a carrot cell culture (taliglucerase alfa) has been compared with mammalian-derived glucocerebrosidase produced in a Chinese hamster ovary cell line. Both variants of the enzyme have been shown to be structurally highly homologous and display comparable enzymatic activity and uptake by macrophages. Furthermore, a single intravenous administration of taliglucerase alfa, at a dose of up to 18 mg/kg corresponding to the 10-fold clinical dose, caused no adverse reactions or clinical or pathological findings in ICR (CD-1®) mice (Shaaltiel et al., 2007).

In addition, allergenicity in humans has been assessed for plant-produced A/Indonesia/05/05 (H5N1) hemagglutinin (HA)-based VLP influenza vaccine candidate, which carries plant-specific α1,3-fucose and β1,2-xylose residues and terminal N-acetylgalcosamine (GlcNAc) that could potentially induce hypersensitivity reactions in vaccinated individuals (Landry et al., 2010). Serological analysis demonstrated that the H5 VLP vaccine did not significantly increase the levels of naturally occurring serum IgG against plant-specific glycans. Furthermore, no subjects in the study had a detectable IgE response to plant glycans before or after vaccination. The authors suggest that terminal GlcNAc could have shielded the proximal α1,3-fucose and β1,2-xylose residues, preventing IgE cross-linking and mast cell/basophil degranulation (Landry et al., 2010). Results of a larger human study with the same vaccine further demonstrated that no vaccinated subject developed IgE directed against MMXF/MUFX motifs, the known allergy-inducing cross-reactive carbohydrate determinants, and no subject showed symptoms of allergy/hypersensitivity (Ward et al., 2014). The authors suggest that plant glycans-specific IgGs may have blocked IgE-mediated allergic responses in vaccinated subjects, contributing to allergen desensitization (Ward et al., 2014).

Plant-specific glycosylation can also be beneficial for some recombinant therapeutic proteins and vaccine antigens. For example, unlike glucocerebrosidase produced in a Chinese hamster ovary cell line, taliglucerase alfa does not need to undergo in vitro glycan processing to expose terminal mannose glycans that are required for the enzyme’s uptake via macrophage mannose receptors (Shaaltiel et al., 2007). Plant N-linked glycans may also serve as glycoadjuvants for mucosal vaccine antigens and facilitate their uptake by antigen-presenting cells (Gomord et al., 2010).

However, there remain concerns that plant-specific glycans may present safety and regulatory issues for the clinical development of some plant-produced pharmaceutical proteins due to the potential immunogenicity of plant glycoepitopes in humans (Bosch et al., 2013; Gomord et al., 2010). To prevent unwanted adverse effects caused by plant-specific glycans, engineered plants have been generated that facilitate mammalian-like glycosylation of recombinant proteins (Bosch et al., 2013; Gomord et al., 2010; Webster and Thomas, 2012). Another approach has been to eliminate N-linked glycans from recombinant proteins in planta by co-expressing a deglycosylating enzyme (Mamedov et al., 2012). Alternatively, putative glycosylation sites can be knocked out by site-directed mutagenesis (Mett et al., 2011).

Strategies for target gene delivery and protein expression in plants include engineering of transgenic or transplastomic plants, based on stable integration of the target gene into the host nuclear or chloroplast genome, respectively, and plant systems for transient expression, some of which are based on replication of recombinant plant viral vectors to boost the nucleic acid template (Daniell et al., 2009; Lico et al., 2012; Rybicki, 2010; Yusibov et al., 2013). Transgenic plants produce recombinant proteins with high efficiency but require relatively long development times to establish producer lines, and the expression of transgenes may decrease over time due to transcriptional gene silencing (Matzke et al., 2000). Transplastomic plants contain abundant copies of a transgene, but the technology is still limited to relatively few species, and there are limitations on post-translational modification in chloroplasts (Daniell et al., 2009; Verma and Daniell, 2007). The transient expression approach is now the most extensively used and allows for the production of large quantities of target proteins within a short time frame (Rybicki, 2010), a particularly attractive feature in the case of pathogens that may be used for bioweapons and for epidemics. Some recombinant proteins produced in plant systems have reached clinical or advanced preclinical stages of development (reviewed in Yusibov et al., 2011; Yusibov et al., 2015). Furthermore, two products have received regulatory approval: a veterinary vaccine against Newcastle disease virus in poultry, produced in transgenic tobacco plant cell suspension by Dow AgroSciences LLC (Indianapolis, IN), approved by the U.S. Department of Agriculture Center for Veterinary Biologics (www.thepoultrysite.com/poultrynews/8949/usda-issues-license-for-plant-cell-produced-newcastle-disease-vaccine-for-chickens), and taliglucerase alfa, the recombinant enzyme indicated to treat patients with Gaucher disease, produced in suspension cultures of a transgenic carrot plant root cell line by Protalix BioTherapeutics Inc. ( Carmiel, Israel), approved by the U.S. Food and Drug Administration (Shaaltiel et al., 2007; Traynor, 2012).

In order for plants to significantly contribute to satisfying the global demand for subunit vaccines, monoclonal antibodies, antivirals and other therapeutic proteins, establishment of high-capacity plant-based manufacturing facilities, operating in compliance with current Good Manufacturing Practices (cGMP) guidelines, will be required. Currently, several medium- and large-scale cGMP-compliant plant facilities are already operating in the North America (e.g. Kentucky BioProcessing [Owensboro, KY], Caliber Biotherapeutics [Bryan, TX], Medicago/Mitsubishi Tanabe Pharma [Quebec, QC, Canada and Research Triangle Park, NC]) and the Fraunhofer USA Center for Molecular Biotechnology [Newark, DE]) and in Europe (e.g. Icon GeneticsNomad Bioscience GmbH [Halle, Germany] and the Fraunhofer Institute for Molecular Biology and Applied Ecology [Aachen, Germany]). Although the total manufacturing capacity of the large-scale facilities is quite significant (e.g. ~40–50 million doses of influenza vaccine per month in less than a month [Yusibov et al., 2015]), establishment of new facilities worldwide and expansion of existing facilities will likely be required to ensure the capability.
for plant-based systems to rapidly respond to an urgent need in case of a pandemic or bioterror threat.

**Advances in the development of plant-based subunit vaccines, monoclonal antibodies and antiviral agents against emerging, reemerging and bioterror-related infections**

Based on the risk to human health, the U.S. Centers for Disease Control and Prevention (CDC) and the National Institute of Allergy and Infectious Diseases (NIAID) established three categories for pathogens that can potentially be used as bioterror agents (http://emergency.cdc.gov/bioterrorism/overview.asp; http://www.niaid.nih.gov/topics/BiodefenseRelated/Biodefense/; http://www.who.int/). Selected pathogens and characteristics of the diseases they cause, for which significant efforts towards plant-based candidate countermeasures have been made, are summarized in Table 1. Pathogens considered by the CDC as bioterror agents include bacteria, viruses and bacteria-released toxins. Category A includes priority pathogens that pose the highest risk to national security and public health, because they can be easily disseminated or transmitted from person to person, result in high mortality rates, might cause public panic and social disruption and require special action for public health preparedness. Category B, the second highest priority group, includes pathogens that are moderately easy to disseminate, result in moderate morbidity rates and low mortality rates and require specific enhancements for diagnostic capacity and enhanced disease surveillance. Category C includes pathogens that could be engineered as bioterrorism agents in the future.

Over the past decade, considerable efforts have been undertaken to produce plant-based subunit vaccines, monoclonal antibodies and other therapeutic proteins to combat emerging, reemerging and bioterror infections. Below, we review the

| Disease (clinical forms) | Agent | Natural reservoir | Type of infection (route of transmission to humans) | Licensed vaccine* |
|-------------------------|-------|------------------|-----------------------------------------------------|------------------|
| **Category A**          |       |                  |                                                     |                  |
| Anthrax (cutaneous, gastrointestinal, inhalational) | *Bacillus anthracis* | Soil, grass-eating animals | Zoonotic (contact with infected materials, inhalation of aerosol; no human to human spread) | Yes |
| Plague (bubonic, septicaemic, pneumonic) | *Yersinia pestis* | Rodents | Zoonotic (flea bite, inhalation of droplets from a person with pneumonic plague) | Discontinued in 1999 |
| Smallpox (ordinary, modified, flat, haemorrhagic) | *Variola major virus* | Humans | Human (prolonged face-to-face contact with an infected person, contact with infected bodily fluids or contaminated objects) | Yes |
| Ebola and Marburg haemorrhagic fevers | Filoviruses | Not identified | Zoonotic (bite of infected fruit bats or primates, contact with virus-contaminated objects, contact with blood or body fluids from a sick person) | No |
| Dengue haemorrhagic fever | *Flavivirus* | Humans | Zoonotic (mosquito bite; no human to human spread) | No |
| Hanta haemorrhagic fever (renal syndrome, pulmonary syndrome) | *Bunyavirus* | Rodents | Zoonotic (contact with infected rodents or their urine and droppings) | No |
| Crimean–Congo haemorrhagic fever | *Bunyavirus* | Mammals, birds | Zoonotic, tick borne (contact with infected wild and domestic animals, contact with body fluids of an infected person) | No |
| Rift Valley haemorrhagic fever | *Bunyavirus* | Rodents | Zoonotic (mosquito and biting fly bites, contact with infected livestock animal tissues; no human to human spread) | No |
| Botulism (classical, infant, wound botulism) | *Toxin of Clostridium botulinum* | Soil and agricultural products | Food borne (ingestion of contaminated food, wound contamination; no human to human spread) | Discontinued in 2011 |
| **Category B**          |       |                  |                                                     |                  |
| West Nile encephalitis | *Flavivirus* | Birds | Zoonotic (mosquito bite; no human to human spread) | No |
| Ricin poisoning | *Toxin of Ricinus communis* | Host plant | Inhalation, ingestion or injection of toxin | No |
| **Category C**          |       |                  |                                                     |                  |
| Yellow fever | *Flavivirus* | Monkeys, humans | Zoonotic (mosquito bite; no human to human spread) | Yes |
| Japanese encephalitis | *Flavivirus* | Pigs, wild birds | Zoonotic (mosquito bite; no human to human spread) | Yes |
| Influenza | Orthomyxoviruses | Mammals including humans and birds (depending on virus strain) | Zoonotic, humans (inhalation or touch of droplets from an infected person or animal) | Yes |
| Severe acute respiratory syndrome, Middle East respiratory syndrome | Coronaviruses | Animals (species not identified) | Zoonotic, humans (inhalation or touch of droplets from an infected person) | No |

*The U.S. FDA.*
literature reporting on the production of such countermeasures, focusing on diseases where most effort has been directed in the plant-based pharmaceutical arena. Plant-produced candidates demonstrating protective efficacy in animal studies are summarized in Table 2, and plant-based countermeasures that are under clinical evaluation are shown in Table 3.

**Anthrax**

Anthrax is a serious infectious disease caused by the Gram-positive, facultative anaerobic, spore-forming bacterium *B. anthracis*, a primary Category A pathogen (Brachman and Kaufmann, 1998; Koch, 1937). Anthrax is a zoonotic disease but can occur in humans after exposure to infected animals or animal tissue (Davies, 1982; Van Ness, 1971) or via bioterrorism (http://www.cdc.gov/anthrax/bioterrorism/index.html; Inglesby *et al.*, 1999; Jernigan *et al.*, 2001). Of three forms of the disease caused by *B. anthracis* – cutaneous, gastrointestinal and inhalational – anthrax caused by inhalation of aerosolized spores is most severe with the highest mortality rates of about 86–89% (Brachman *et al.*, 1960) and, therefore, most important in the context of bioterrorism. The principal virulence factor of *B. anthracis*, B3-kDa PA, forms two exotoxins, lethal toxin (LeTx) and oedema toxin, via binding with LF or EF, respectively, which enter the cytoplasm leading to the cell death (Young and Collier, 2007). PA elicits specific neutralizing antibodies that correlate with protective immunity (Fowler *et al.*, 1999; Little *et al.*, 1997, 2004; Pitt *et al.*, 2001; Weiss *et al.*, 2006) and is the major target for anthrax vaccine development. The United States approved anthrax vaccine, BioThrax® (Anthrax Vaccine Adsorbed), is based on the PA protein prepared from cell-free filtrates of microaerophilic cultures of an avirulent, nonencapsulated strain of *B. anthracis*, formulated with aluminum hydroxide adjuvant (Emergent BioSolutions, 2012). To achieve full protective efficacy, BioThrax® should be administered in multiple doses (as many as five doses over a period of 18 months and at 1 year intervals thereafter) and may cause safety issues due to lot-to-lot variations in the composition (Emergent BioSolutions, 2012).

Over the last decade, significant efforts have been made towards the development of plant-based orally administered vaccines against anthrax. Initially, attempts have been made to produce anthrax subunit vaccine candidates in transgenic plants such as tomato, potato and mustard (Aziz *et al.*, 2002, 2005; Gorantala *et al.*, 2014). For example, full-length PA (PA83) was stably expressed in transgenic tobacco plants and demonstrated a cytotoxic activity similar to native PA in vitro when total soluble protein extracted from the transformed leaves was added to macrophage-like cells in combination with LF (Aziz *et al.*, 2002). Immunogenicity of this vaccine candidate was not evaluated. Subsequently, PA83 was expressed in transgenic tomato plants and, when administered intraperitoneally as total soluble protein extracted from the transgenic tomato leaves, elicited LeTx-neutralizing antibodies in mice. However, the transgene expression levels as well as the titres of antibodies raised were low (Aziz *et al.*, 2005). More recently, an attempt was made to develop a comprehensive orally administered vaccine suitable for both veterinary and human use. For this purpose, PA83 was expressed in transgenic mustard, *Brassica juncea*, whose leaves and stems can be consumed as a vegetable by humans and used as feed for cattle, although the expression levels and in vitro cytotoxicity of this PA83 varied due to random total soluble protein from transgenic *B. juncea*, adjuvanted with Alhydrogel or cholera toxin (CT) and generated significant anti-PA IgG titres (Gorantala *et al.*, 2014). Significant serum IgA titres and faecal secretory IgA titres were also detected, indicating the development of mucosal immune responses. Oral immunization with either adjuvanted or nonadjuvanted PA83 from *B. junece* conferred 60% survival on mice following lethal intraperitoneal anthrax challenge (Gorantala *et al.*, 2014).

An LF-based anthrax vaccine candidate has also been engineered and produced in transgenic plants. A nontoxic domain 1 of LF, fused to cholera toxin B subunit (CTB), was produced in transgenic potatoes (Kim *et al.*, 2004). CTB, a nontoxicogenic pentameric subunit of CT, is a highly efficient carrier for mucosal antigen delivery of chemically or genetically coupled antigens and acts via binding to ganglioside GM1 receptors on the surface of intestinal epithelial cells (Holmgren *et al.*, 1993). This facilitates uptake of the ligand–antigen conjugate and stimulates antigen presentation and mucosal immune responses. CTB expressed in transformed tobacco chloroplasts has been confirmed to form pentamers and share the biological activity of native CTB (Daniell *et al.*, 2001). The CTB–LF fusion protein synthesized in potato tuber tissue has also been shown to assemble into pentamers that could bind to ganglioside GM1 (Kim *et al.*, 2004). Immunogenicity of this orally administered vaccine candidate has not, however, been evaluated.

To increase the expression levels of vaccine antigen, chloroplast transformation of tobacco plants (Chebolu and Daniell, 2009) has been pursued. Using optimized chloroplast protein expression conditions, an increased expression of PA83 compared with nuclear transgenic tobacco plants has been achieved (Aziz *et al.*, 2005). The chloroplast-derived PA protein exhibited a potent cytotoxic effect against a macrophage cell line in vitro in the presence of LF. Similar to PA83 from transgenic *B. junece*, chloroplast-derived PA83 generated significant serum anti-PA IgG and IgA titres following oral administration with CT adjuvant, indicating the development of both systemic and mucosal immune responses (Gorantala *et al.*, 2014). Furthermore, orally immunized mice demonstrated 80% survival after lethal anthrax challenge (Gorantala *et al.*, 2014). In addition to full-length PA, its fragment, PA domain 4 (PA(dIV)), has also been expressed in tobacco chloroplasts and the expression levels were found to be significantly higher compared with nuclear transformation (up to 5.3% vs. 0.8% of total soluble protein) (Gorantala *et al.*, 2011). Both intraperitoneal and oral immunizations with total soluble protein from the PA(dIV) transplastomic plants elicited antibody titres of >10^4, similar to *Escherichia coli*-derived PA(dIV), which were predominantly of the IgG1 subtype and possessed toxin-neutralizing activity in vitro. In addition, intraperitoneal and oral immunization with chloroplast-derived PA(dIV), adjuvanted with Alhydrogel or CT, respectively, each conferred 60% protection of mice against lethal anthrax challenge, respectively, compared with 100% and 80% protection with adjuvanted *E. coli*-derived PA(dIV). Further evaluation of mucosal responses following oral immunizations detected low secretory IgA titres in faecal extracts from mice receiving adjuvanted plant- or *E. coli*-derived PA(dIV). In addition, anti-PA(dIV) antisera enhanced *B. anthracis* spore uptake by macrophages in vitro and suppressed spore germination (Gorantala *et al.*, 2011).

Daniell and colleagues have successfully introduced the full-length PA gene into the chloroplast genome of tobacco plants and achieved the expression levels of up to 2.5 mg of PA83 per gram of fresh leaf tissue with target stability in leaves or crude plant extract for several months. Chloroplast-derived PA83 was cytotoxic against mouse macrophages in vitro in the presence of
| Plant-produced candidate countermeasures demonstrating protective efficacy in preclinical studies |
|-------------------------------------------------|
| **Product type**<br>**(name)** | **Target antigen** | **Recombinant product detail** | **Host plant** | **Expression strategy** | **Route of immunization**<br>**(adjuvant)** | **Form of antigen** | **Efficacy (species, route of challenge)** | **References** |
| -------------------------- | ----------------- | ------------------------ | ------------- | --------------------- | ----------------- | -------------- | ----------------------------- | ------------------ |
| Anthrax<br>SUV | PA | PA83 | *Brassica juncea* | Transgenic | Oral (CT or none) | Crushed leaf material | 60% survival (mice, IP) | Gorantala et al. (2014) |
|  | PA | PA83 | *Nicotiana tabacum* | Transplastomic | IP (AL) | TSP | 80% survival (mice, IP) | Gorantala et al. (2014) |
|  | PA | DIV | *N. tabacum* | Transplastomic | IP (AL) TSP | 80% survival (mice, IP) | Gorantala et al. (2011) |
|  | PA | PA83 | *N. tabacum* | Transplastomic | SC (AL) Purified | 100% survival (mice, IP) | Koya et al. (2005) |
|  | PA | PA83 | *Nicotiana benthamiana* | Transient | IM (AL) Purified | 100% survival (rabbits, IN) | Chichester et al. (2013) |
| mAb | PA | Glycosylated, full size | *N. benthamiana* | Transient | IP (none) Purified | 90% survival (mice, IP) | Mett et al. (2011) |
|  | PA | Nonglycosylated, full size | *N. benthamiana* | Transient | IP (none) IV (none) Purified | 100% survival (mice, IP) | Mett et al. (2011) |
| Plague<br>SUV | F1, LcrV | F1–LcrV fusion | *Lycopersicon esculentum* | Transgenic | Oral (CT) | 50% survival (mice, SC) | Alvarez and Cardineau (2010) |
|  | F1, LcrV | F1–LcrV fusion | *N. tabacum* | Transplastomic | SC (AL) prime and boost | 33% survival (mice, IN) | Arlen et al. (2008) |
|  | F1, LcrV | Stand-alone LcrV | *N. benthamiana* | Transient | SC (AL) | 88% survival (mice, IN) | Santi et al. (2006) |
|  | F1, LcrV | Combined individual LicKM fusions | *N. benthamiana* | Transient | SC (AL) | 75% survival (guinea pigs, IN) | Mett et al. (2007) |
|  | F1, LcrV | Dual LicKM fusion | *N. benthamiana* | Transient | SC (AL + Quil A) | 100% survival (NHPs, IN) | Chichester et al. (2009b) |
| Smallpox<br>SUV | B5 | Extracellular antigenic domain | *N. tobacco* | Transgenic | IM (CpG/AL) | 100% survival (mice, IN) | Golovkin et al. (2007) |
| Ebola<br>mAb (MB-003) | GP | Triple cocktail (13C6, 13F6, 6D8), humanized, glycoengineered | *N. benthamiana* | Transient | IV (none) Purified | 43%–100% survival depending on day of challenge (NHPs, IM) | Olinger et al. (2012); Pettitt et al. (2013) |
| mAb (ZMapp) | GP | Triple cocktail (13C6, 2G4, 4G7), humanized, glycoengineered | *N. benthamiana* | Transient | IV (none) Purified | 100% survival (NHPs, IM) if given up to 5 days postchallenge | Qiu et al. (2014) |
| Product type | Target antigen | Recombinant product detail | Host plant | Expression strategy | Route of immunization (adjuvant) | Form of antigen | Efficacy (species, route of challenge) | References |
|--------------|----------------|-----------------------------|------------|--------------------|----------------------------------|-----------------|-------------------------------------|------------|
| SUV          | GP             | 6D8 IgG-GP1 fusion, immune complex | N. benthamiana | Transient          | SC (PI:PC)                       | Purified        | 80% survival (mice, IP)             | Phoolcharoen et al. (2011) |
| West Nile mAb (hE16) | E DIII | Humanized, full size | N. benthamiana | Transient          | IP (none)                        | Purified        | 80%-90% survival (mice, SC) depending on prophylactic or therapeutic regimen | Lai et al. (2010) |
| mAb (hE16)  | E DIII | Humanized monomeric scFv-C, and tetravalent scFv-C,–scFv-C, fusions | N. benthamiana | Transient          | IP (none)                        | Purified        | 70%-80% survival (mice, SC) depending on prophylactic or therapeutic regimen | He et al. (2014a,b) |
| mAb (hE16) | E DIII | Humanized, glycoengineered, full-size stand-alone and scFv-C, fusion | N. benthamiana | Transient          | IP (none)                        | Purified        | 70%-100% survival (mice, SC) depending on mAb variant and prophylactic or therapeutic regimen | Lai et al. (2014) |
| Influenza SUV | H3N2 HA, NA | Combination of HA-SD/HA-GD, LicKM fusions | N. benthamiana | Transient          | SC (AL)                          | Purified        | 100% no virus titre in nasal washes (ferrets, IN) | Mett et al. (2008) |
| SUV          | H5N1 HA       | Monomer                     | N. benthamiana | Transient          | SC (Quil A)                      | Purified        | 100% survival (ferrets, IN)         | Shoji et al. (2009) |
| SUV          | H1N1 HA       | Monomer and trimer          | N. benthamiana | Transient          | IM (AL)                          | Purified        | 100% survival (mice, IN)           | Shoji et al. (2013) |
| SUV          | H5N1 HA       | eVLP                        | N. benthamiana | Transient          | IM (AL)                          | Purified        | 100% survival (mice, IN)           | Landry et al. (2010) |
| Severe acute respiratory syndrome Antiviral (griffithsin) | S | Lectin, homodimer | N. benthamiana | Transient          | IN (none)                        | Purified        | 100% survival (mice, IN) when given before challenge | O’Keefe et al. (2009, 2010) |

AL, Alhydrogel; C\textsubscript{\textalpha}, constant domains of immunoglobulin heavy chain; C\textsubscript{\textgamma}, constant domain of immunoglobulin light chain; CT, cholera toxin; DIII, domain III; DIV, domain IV; GD, globular domain; GP, glycoprotein; IC, intracranial; IM, intramuscular; IN, intranasal; IP, intraperitoneal; IV, intravenous; mAb, monoclonal antibody; NA, neuraminidase; NHPs, nonhuman primates; PA, protective antigen; PI:PC, polyinosinic polycytidylic acid; SC, subcutaneous; scFv, single-chain variable fragment of immunoglobulin; SD, stem domain; SUV, subunit vaccine; TSP, total soluble protein; eVLP, enveloped virus-like particle; VN, virus neutralizing.
| Company/organization | Product type (name)* | Product detail | Target antigen/strain | Adjuvant (number of doses) | Route of administration | Participant condition | Product development status | Reference or clinical trial identifier (NCT)† |
|----------------------|----------------------|---------------|-----------------------|---------------------------|------------------------|-----------------------|---------------------------|-----------------------------------------------|
| LeafBio mAb (ZMapp) | mAb (ZMapp)          | Triple cocktail (13C6, 2G4, 4G7), humanized, glycoengineered | GP/Zaire | None (3) | IV | Infected | Investigational therapy (five of seven treated patients survived) | McCarthy (2014); Lyon et al. (2014) |
| LeafBio mAb (ZMapp) | mAb (ZMapp)          | Triple cocktail (13C6, 2G4, 4G7), humanized, glycoengineered | GP/Zaire | None (not specified) | IV | Infected | Phase 1/2 (enrolling by invitation) | NCT02363322 |
| LeafBio mAb (ZMapp) | mAb (ZMapp)          | Triple cocktail (13C6, 2G4, 4G7), humanized, glycoengineered | GP/Zaire | None (1) | IV | Healthy | Phase 1 (recruiting) | NCT02389192 |
| FhCMB SUV (PA83-FhCMB) | Monomer | PA83 | Alhydrogel (3) | IM | Healthy | Phase 1 (recruiting) | NCT02239172 |
| FhCMB SUV (HAC1) | Monomer | HA/H1N1 seasonal | Alhydrogel (2) | IM | Healthy | Phase 1 (completed) | NCT01177202 |
| FhCMB SUV (HAI-05) | Monomer | HA/H5N1 pandemic | Alhydrogel (2) | IM | Healthy | Phase 1 (completed) | NCT01250795 |
| Medicago SUV | eVLP | HA/H1N1 seasonal | None (1) | IM | Healthy | Phase 1 (completed) | NCT01302990 |
| Medicago SUV | eVLP | HA/H1N1, H3N2, B seasonal | None (1) | IM | Healthy | Phase 1/2 (ongoing) | NCT01991587 |
| Medicago SUV | eVLP | HA/H1N1, H3N2, B seasonal | None (1) | IM | Healthy | Phase 1 (recruiting) | NCT02233816 |
| Medicago SUV | eVLP | HA/H1N1, H3N2, B seasonal | Alhydrogel (1) | IM | Healthy | Phase 1 (completed) | NCT01991561 |
| Medicago SUV | eVLP | HA/H5N1 pandemic | Alhydrogel (2) | IM | Healthy | Phase 1 (completed) | NCT00984945 |
| Medicago SUV | eVLP | HA/H5N1 pandemic | Alhydrogel (2) | IM | Healthy | Phase 2 (completed) | NCT01244867 |
| Medicago SUV | eVLP | HA/H5N1 pandemic | Alhydrogel (2) | IM | Healthy | Phase 2 (ongoing) | NCT01244867 |
| Medicago SUV | eVLP | HA/H5N1 pandemic | GLA-SE (2) | IM | Healthy | Phase 2 (ongoing) | NCT01991561 |
| Medicago SUV | eVLP | HA/H5N1 pandemic | GLA-SE (2) | IM | Healthy | Phase 2 (ongoing) | NCT01991561 |
| Medicago SUV | eVLP | HA/H5N1 pandemic | GLA-AF (1) | IM and ID | Healthy | Phase 1 (completed) | NCT01657929 |
| Medicago SUV | eVLP | HA/H7N9 pandemic | Alhydrogel (2) | IM | Healthy | Phase 1 (ongoing) | NCT02022163 |

*All listed plant-based products were transiently produced in Nicotiana benthamiana plants.
†Registered at https://clinicaltrials.gov.
eVLP, enveloped virus-like particle; FhCMB, Fraunhofer USA Center for Molecular Biotechnology; GLA-SE, glucopyranosyl lipid adjuvant in squalene emulsion; GLA-AF, GLA aqueous nanoparticle suspension formulation; GP, recombinant glycoprotein; HA, recombinant hemagglutinin; ID, intradermal; IM, intramuscular; IV, intravenous; mAb, monoclonal antibody; PA, protective antigen; SUV, subunit vaccine.
peptide-N-glycosidase F (PNGase F). Previously, this strategy was used to produce LF IgG1 as well as LeTx-neutralizing antibodies (Chichester et al., 2007; Shoji et al., 2009). Mice immunized with this dual anthrax vaccine candidate adjuvanted with complete or incomplete Freund’s adjuvant developed high titres of anti-PA and anti-LF IgG1 as well as LeTx-neutralizing antibodies (Chichester et al., 2007). Using the same expression system, a single-component anthrax vaccine candidate was subsequently produced in Nicotiana benthamiana plants, comprising nonfused, full-length, glycosylated, recombinant PA83 (Chichester et al., 2013). This new vaccine elicited high titres of toxin-neutralizing antibodies in mice and rabbits after two administrations in the presence of Alhydrogel. Furthermore, rabbits that received the adjuvanted PA83 were completely protected from a lethal aerosolized B. anthracis challenge. The vaccine antigen formulated with Alhydrogel was found to be stable and retained immunogenicity after 2-week storage at 4 °C, which is suitable for clinical use. Safety and immunogenicity of the plant-produced PA83 in healthy adults aged 18–49 years are currently being evaluated in a Phase 1 clinical trial (NCT02239172) (https://clinicaltrials.gov/ct2/show/NCT02239172?term=pa83&rank=1), in which immunogenicity of the vaccine candidate is being assessed as changes in serum antibody titres from baseline after three intramuscular immunizations at four dose levels in the presence of Alhydrogel.

As native PA of B. anthracis is not a glycoprotein but contains potential N-linked glycosylation sites, glycosylation of recombinant PA during expression in eukaryotic systems may negatively affect biological activity of this antigen. For example, pp-PA83 was not able to combine with LF, form LeTx and induce cell death in vitro (Mamedov, T., Chichester, J.A., Jones, R.M., Ghosh, A., Coffin, M.V., Herschbach, K., Prokhnevsky, A.I., Streatfield, S.J., Yusibov, V., in preparation.). To eliminate the negative impact of aberrant glycosylation, a nonglycosylated version of PA83 was produced in Nicotiana benthamiana plants by co-expressing bacterial peptide-N-glycosidase F (PNGase F). Previously, this strategy was proven efficient, as co-expression of PNGase F resulted in vivo deglycosylation of Pf48/45, a sexual stage antigen from P. falciparum, in N. benthamiana plants (Mamedov et al., 2012). In planta deglycosylated pp-PA83, pp-dPA83, regained the ability to form LeTx in vitro and induced significantly higher levels of toxin-neutralizing antibody responses in mice compared with glycosylated pp-PA83. Besides soluble monomeric protein-based subunit vaccines, VLP-based anthrax vaccine candidates have also been produced in plants. For example, genetic fusion of a 15-amino acid epitope from domain 4 of PA (PA-D4) into alfalfa mosaic virus coat protein (AlMV CP) and introduction of this construct into the P12 strain of Nicotiana tabacum cv. Samsun NN plants (expressing AlMV P1 and P2 replicase genes) resulted in high yields of the fusion protein and its assembly into chimeric VLPs (Brodzik et al., 2005). Two intraperitoneal immunizations of mice with these purified recombinant VLPs, in the presence of complete (for the first immunization) or incomplete (for the second immunization) Freund’s adjuvant, elicited serum antibodies reacting with PA; however, a detectable anthrax-specific antibody response was observed in only two of five immunized mice. In another study by the same group, hepatitis B core protein (HBcAg) carrying PA-D4 was expressed in transgenic low-alkaloid N. tabacum cv. LAMD609 plants (Bandurska et al., 2008). Electron microscopy did not detect formation of VLPs by this recombinant chimeric protein, in contrast to unconjugated HBcAg. Immunogenicity of the purified protein in mice was evaluated following three intramuscular injections in the presence of adjuvant (combination of CpG oligodeoxynucleotides and Alhydrogel). Analysis of immunized mouse sera demonstrated the presence of significant anti-PA IgG titres, which were predominantly IgG2a/IgG2b subtypes, consistent with the serum IgG profile of mice immunized with PA83 (Bandurska et al., 2008). Neither AlMV CP- nor HBcAg-based plant-produced vaccine candidates were assessed for protective efficacy.

The plant transient agroinfiltration-based expression approach has also been utilized to produce protective monovalent monoclonal antibodies against anthrax PA. For example, a human anti-PA monoclonal antibody was produced in N. benthamiana plants, and its LeTx-neutralizing activity was demonstrated in vitro and in vivo, being comparable to that of a hybridoma-produced anti-PA monoclonal antibody (Hull et al., 2005). In a subsequent study, both glycosylated and nonglycosylated (PANG) versions of the anti-PA monoclonal antibody were produced in N. benthamiana plants (Mett et al., 2011). Both glycosylated monoclonal antibody and PANG monoclonal antibody neutralized anthrax LeTx activity in vitro and protected mice against an intraperitoneal challenge with spores of B. anthracis Sterne strain (90% and 100% survival, respectively). When tested in nonhuman primates (NHPs), the PANG monoclonal antibody was superior to the glycosylated anti-PA monoclonal antibody, exhibiting a significantly longer terminal half-life and 100% protection (vs. 40% protection for the glycosylated anti-PA monoclonal antibody) against challenge with a lethal dose of aerosolized anthrax spores. These data demonstrate the potential of the plant-produced PANG monoclonal antibody as a therapy for the treatment of inhalational anthrax.

Plague

Plague, the cause of major pandemics and epidemics throughout human history, is caused by a Category A Gram-negative, non-spore-forming, intracellular bacterium Y. pestis whose life cycle includes fleas and small mammals, primarily rodents (www.niaid.nih.gov/topics/plague). Numerous data suggest that Y. pestis recently evolved from the enteric bacterium Yersinia pseudotuberculosis that adapted to an insect vector (the flea) and transmission between mammalian hosts via subcutaneous and pneumonic routes. A common route of bacterial transmission from animal to animal is by the bite of an infected flea. After the flea ingests blood containing Y. pestis from a rodent host, the bacterium multiplies in the flea gut, resulting in blood clotting in...
the proventriculus and blocking the food (blood) passage from the oesophagus to the midgut. During feeding, the blocked flea regurgitates infected blood into the bloodstream of the mammal, transmitting the bacterium to the next host (Prentice and Rahalison, 2007). Transmission of the pathogen to humans may occur through the flea bite, contact with fluids from an infected animal or aspiration of respiratory droplets from an infected animal or an individual with pneumonic lesions. Depending on how the bacterium enters the body, plague can manifest in three clinical forms, bubonic (the most common form of naturally occurring plague), septicemic or pneumonic, with pneumonic plague being the most virulent and fatal form of the disease (Cobb and Chansolme, 2004; Inglesby et al., 2000; Perry and Fetherston, 1997; www.niaid.nih.gov/topics/plague). A formalin-killed whole-cell Y. pestis vaccine against plague was the only plague vaccine licensed in the United States, but it was not effective against pneumonic plague and its manufacture was discontinued in 1999 (Titball and Williamson, 2004).

Subunit vaccines against plague are based on two Y. pestis antigens that are either expressed individually and combined or produced as a fusion protein: the antiphagocytic fraction 1 capsular envelope glycoprotein (F1) and the low calcium response virulent protein (LcrV) (Prentice and Rahalison, 2007). Fusion protein (F1–LcrV)-based orally administered plague subunit vaccine candidates have been produced by generating F1–LcrV transgenic plants such as tomato (Alvarez et al., 2006), lettuce (Rosales-Mendoza et al., 2010) and carrot tap root (Rosales-Mendoza et al., 2011), and their immunogenicity following either oral or subcutaneous administration has been evaluated. For example, mice were subcutaneously primed with bacteria-produced F1–LcrV and then orally boosted with several doses of freeze-dried F1–LcrV transgenic tomato fruits along with bacterial CT as an adjuvant. The immunized mice were shown to generate antigen-specific serum IgG and intestinal IgA (Alvarez et al., 2006). When these immunized mice were subcutaneously challenged with a 20 x LD50 dose of Y. pestis, protection was observed in 50% of the animals, compared with 20% when mice were boosted with nontransgenic tomato (Alvarez and Cardineau, 2010). The authors explained the observed medium level of mouse protection by the extended time interval allowed between the last vaccine boost and the bacterial challenge (18 months), which, according to a previous report (Fujihashi et al., 2000), may have resulted in a reduced ability of these aged mice to elicit systemic and mucosal antigen-specific immune responses. On the other hand, the results indicate that protection against Y. pestis was maintained over long term. The level of target accumulation originally obtained in the freeze-dried transgenic tomato tissue was high (4–10% total soluble protein) (Alvarez et al., 2006); however, it was subsequently increased (up to 16% total soluble protein) by means of P19-induced suppression of RNA-mediated target gene silencing (Alvarez et al., 2008).

Subcutaneous immunization of mice with total protein extract from F1–LcrV transgenic lettuce, adsorbed on Alhydrogel, also elicited significant levels of specific serum IgG, although the F1–LcrV expression level was low (0.08% of total soluble protein) (Rosales-Mendoza et al., 2010). Similarly, three subcutaneous injections of mice with soluble protein extract from freeze-dried F1–LcrV transgenic carrot tap roots elicited considerable levels of antigen-specific serum IgG (Rosales-Mendoza et al., 2011). With all three transgenic plant-produced plague subunit vaccine candidates, IgG1 was a predominant IgG isotype produced, indicating Th2-type immune responses (Alvarez et al., 2006; Rosales-Mendoza et al., 2010, 2011). Protective efficacy of these plague vaccine candidates has not been reported.

F1–LcrV has also been expressed in transformed tobacco chloroplasts, achieving up to 14.8% of total soluble protein (Arlen et al., 2008). Crude extract from these transplastomic tobacco leaves was lyophilized and enriched for animal immuno- genicity studies. Mice that were primed subcutaneously with Alhydrogel-adsorbed F1–LcrV and then repeatedly boosted orally with unadjuvanted F1–LcrV generated substantial titres of antigen-specific serum IgG of predominantly IgG1 isotype, which were higher than in mice that were subcutaneously boosted with adjuvanted F1–LcrV. Antigen-specific serum IgA titres were also detected in F1–LcrV-boosted mice. Among orally boosted mice, 88% were protected against lethal aerosolized Y. pestis challenge, compared with 33% of mice that were subcutaneously boosted, indicating that oral booster with F1–LcrV elicited protective immune responses (Arlen et al., 2008). Protection predominantly correlated with circulating IgG1 (r = 0.71) rather than IgA antibody titres (r = 0.26) (Arlen et al., 2008).

Examples of transiently produced plague subunit vaccines include F1 and LcrV antigens and a F1–LcrV fusion expressed in N. benthamiana plants using the MagnICON™ deconstructed vectors (Santi et al., 2006). All these vaccine antigens, injected subcutaneously in three doses into guinea pigs, elicited antigen-specific serum IgG and conferred significant protection against an aerosol challenge with virulent Y. pestis at 21 days postexposure, with the highest survival rate (six of eight animals) for LcrV vaccination, followed by F1–LcrV vaccination (five of eight animals) (Santi et al., 2006). These plant-produced plague antigens weakly activated innate immune cells (human monocytes), but induced antigen-specific T-cell and antibody responses similar to those occurring during Y. pestis infection (Del Prete et al., 2009).

F1 and LcrV antigens were also produced in N. benthamiana plants as fusions to the LicKM carrier (Musiychuk et al., 2007), either single [F1–LicKM and LcrV–LicKM (Mett et al., 2007)] or dual [F1–LcrV–LicKM (Chichester et al., 2009b)]. Three subcutaneous injections of combined F1–LicKM and LcrV–LicKM, adjuvanted with Alhydrogel, elicited strong serum IgG and IgA titres and conferred complete protection on Cynomolgus macaques against lethal challenge with Y. pestis (Mett et al., 2007). Similarly, F1–LcrV–LicKM, adjuvanted with Alhydrogel and Quil A and administered subcutaneously in three doses, induced high titres of serum IgG, predominantly of the IgG1 isotype, against both F1 and LcrV. F1–LcrV–LicKM also fully protected Cynomolgus macaques against lethal challenge with aerosolized Y. pestis (Chichester et al., 2009b), suggesting the strong potential as a vaccine candidate against pneumonic plague.

Smallpox

Smallpox, a disfiguring and highly contagious infectious disease affecting human populations since ancient times, is caused by the Variola virus, a Category A pathogen. Symptoms appear 12–14 days after infection and include fever, malaise, headache, prostration, severe back pain, and sometimes abdominal pain and vomiting. Two to three days later, the body temperature falls and a rash appears, primarily on the face, hands, forearms and the trunk. Historically, the mortality of ordinary smallpox is approximately 30%. Transmission of the virus occurs through inhalation of aerosols or droplets from infected symptomatic people. Due to
the WHO-initiated global immunization campaign, smallpox was declared eradicated in 1980 and routine vaccinations have been stopped (Fenner et al., 1988; www.niaid.nih.gov/topics/smallpox).

The risk for smallpox occurring as a result of bioterrorism is considered very low. However, high-risk groups, such as scientists and medical professionals working with smallpox and similar viruses in a research setting, and military personnel, are still required to receive the vaccine. Following recommendations of the Advisory Committee on Immunization Practices and the National Vaccine Advisory Committee, sufficient amounts of smallpox vaccine doses are now available to vaccinate every person in the United States, if needed. The licensed smallpox vaccine is based on live vaccinia virus and is highly effective (http://www.bt.cdc.gov/agent/smallpox/vaccination/facts.asp; http://www.bt.cdc.gov/agent/smallpox/prep/cdc-prep.asp).

Smallpox subunit vaccine development relies on several orthopoxvirus antigens that elicit neutralizing antibody responses. For example, B5, the protein of the extracellular enveloped vaccinia virus, is the main target of neutralizing antibodies present in vaccinia gamma-globulin of immunized humans (Bell et al., 2004). Furthermore, B5 is a potent inducer of immune responses shown to protect mice and NHPs against poxvirus infections (Benhnia et al., 2009; Buchman et al., 2010).

The full extracellular antigenic domain (amino acids 20–275) of the B5 protein was produced in soluble and insoluble forms by transient and stable expression in plants (Golovkin et al., 2007). Transient expression in N. benthamiana, based on Magnifection™ (Gleba et al., 2005), resulted in rapid production of soluble B5 to high yields. Nuclear transformation of tobacco and collard resulted in stable production of soluble and insoluble forms of B5, respectively. The authors observed major differences in plant-produced B5 (pB5) immunogenicity depending on protein solubility and route of administration. Mice were immunized via oral, intranasal or parenteral routes. For oral immunization, the animals received B5-transgenic fresh collard leaves or lyophilized and powdered leaf pellets, with or without CT as a mucosal adjuvant, by mouth, or purified (>50%) pB5 with CT through gavage. No B5-specific IgG titres were detected in mouse sera even in the presence of the CT adjuvant, despite the presence of systemic and intestinal anti-CT antibodies. For intranasal immunization, mice were administered three doses of purified pB5, with or without CT adjuvant. In the presence of CT, purified pB5 elicited B5-specific serum IgG. Parenteral immunization with purified pB5 was performed by two subcutaneous injections with Freund’s adjuvant and one intraperitoneal injection without adjuvant and resulted in the highest anti-B5 IgG titres. Furthermore, when purified pB5 was given to mice in three intramuscular injections in the presence of the CpGAlum adjuvant combination, it induced anti-B5 serum IgG titres that reduced vaccinia virus spread in the comet inhibition assay and conferred protection against a lethal challenge with vaccinia virus (Golovkin et al., 2007).

In a subsequent study, the authors confirmed the efficiency of intranasal vaccination with purified pB5 adjuvanated with CT, demonstrating that at sixfold higher doses, it could elicit systemic IgG titres comparable to those induced by intramuscular vaccination. In contrast to intramuscular immunization, intranasal vaccination induced not only B5-specific serum IgG but also IgA in the respiratory tract, which was dose dependent. The analysis of IgG isotypes demonstrated balanced IgG1/IgG2a (Th1/Th2) anti-B5 responses, essential for protection against vaccinia virus (Matsui et al., 2005; Reading and Smith, 2003), after both intramuscular and intranasal vaccination. Contamina-

tion of purified pB5 with increased amounts of total soluble protein from wild-type tobacco plants decreased B5-specific antibody responses, especially those induced by intranasal immunization, and shifted them towards the Th2 type. Taken together, these data indicate the importance of antigen dose and purity for efficient intranasal vaccination (Portocarrero et al., 2008).

Several other vaccinia virus antigens, including A27L, A33R and L1R, have been expressed in transplastomic tobacco plants; however, no immunogenicity studies of these plant-produced antigens have been reported (Rigano et al., 2009a,b).

Viral haemorrhagic fevers

Viral haemorrhagic fevers (VHF) are acute diseases caused by small RNA viruses including filoviruses (Ebola, Marburg), flaviviruses (dengue, yellow fever), arenaviruses (Junin, Machupo, Guanarito, Chapare, Lassa and Lujo) and bunyaviruses (Crimean-Congo, Hanta, Rift Valley). Viruses associated with most VHF vaccines are zoonotic and naturally reside in animal reservoir hosts or arthropod vectors. Humans can become infected through contact with infected animal tissues or inhalation of aerosolized excreta. For some VHF vaccines, such as Ebola, Marburg, Lassa and Crimean–Congo viruses, transmission from person to person may also occur, through contact with infected blood or body secretions. Specific signs and symptoms vary by the type of VHF, but initial symptoms of VHFs often include fever, fatigue, dizziness, muscle aches, loss of strength and exhaustion. While the disease can be very mild, patients with severe cases of VHFs often show signs of the overall vascular system damage, including bleeding. Severely ill patients may also show shock, nervous system malfunction, coma, delirium, seizures and kidney failure (http://www.cdc.gov/ncidod/dvrd/sbp/mnpages/dispages/Fact_Sheets/Viral_Hemorrhagic_Fevers_Fact_Sheet.pdf; Borio et al., 2002; Weber and Rutala, 2001). Natural outbreaks of VHFs occur periodically in endemic areas. For example, the recent epidemic of Ebola fever in several West African countries (Frieden et al., 2014; http://www.cdc.gov/vhf/ebola/outbreaks/2014-west-africa/previous-updates.html) with reported case fatality rates of up to 71% among the general population and 57–59% among hospitalized patients (WHO Ebola Response Team, 2014; http://apps.who.int/ebola/en/status-outbreak/situation-reports/ebola-situation-report-31-December-2014; http://apps.who.int/ebola/en/current-situation/ebola-situation-report; http://www.cdc.gov/ncidod/dvrd/sbp/mnpages/dispages/vhf.htm).

Among VHF vaccines, yellow fever and Argentine haemorrhagic fever are the only two infections for which vaccines have been approved (http://www.cdc.gov/ncidod/dvrd/sbp/mnpages/dispages/vhf.htm). The vaccine against Argentine haemorrhagic fever is a live attenuated Junin virus vaccine registered and manufactured in Argentina. The vaccine demonstrated an optimal balance between reactogenicity and immunogenicity (Ambrosio et al., 2011). Currently, there are four producers of yellow fever vaccines that are prequalified by the World Health Organization to supply vaccines to international agencies: Bio-Manguinhos/Fiocruz (17DD-YFV, Brazil), Sanofi-Pasteur (YF-Vax®, France), Institute Pasteur in Dakar (Senegal) and Chumakov Institute of Poliomyelitis and Viral Encephalitides (Russian Federation). All currently licensed yellow fever vaccines comprise a live attenuated YF virus (strain 17D) produced in embryonated hens’ eggs (http://www.who.int/immunization_standards/vaccine_quality/PQ_vaccine_list_en). The YF-Vax® vaccine (Sanofi Pasteur, Swiftwater, PA), licensed in the United States, is highly effective, with a
single dose being sufficient to confer sustained immunity and lifelong protection against the disease, providing effective immunity within 30 days for 99% of persons vaccinated (Sanofi Pasteur, 2013; http://www.who.int/mediacentre/factsheets/fs100/en). Although no vaccine against dengue has been approved yet, a vaccine candidate, ChimeriVax™-Dengue (Sanofi Pasteur, Lyon, France), is undergoing advanced clinical evaluation (Sinha, 2014). ChimeriVax™-Dengue is a tetravalent vaccine composed of four YF virus-based recombinant chimeric live attenuated viruses carrying glycoproteins (GPs) of four dengue virus (DENV) serotypes (Guy et al., 2011).

Ebola fever

Ebola virus is a Category A pathogen. Most current investigational therapies against Ebola target the major virulence factor of Ebola virus (EBOV), GP, the transmembrane molecule that mediates Ebola virus infection in rhesus macaques, 43% of animals still survived the lethal challenge with EBOV. Furthermore, MB-003 was partially protective (four of six monkeys) if the treatment was given 24 or 48 h postinfection, whereas no monkeys survived in the untreated group. In addition, plant-produced MB-003 was three times as potent as the Chinese hamster ovary cell-produced recombinant counterpart monoclonal antibodies (Olinger et al., 2012). The difference is suggested to be due to the absence of the core fucose on the Fc region of the plant-derived monoclonal antibodies leading to increased antibody binding to the Fc receptor gamma III and enhanced antibody-dependent cellular cytotoxicity compared with monoclonal antibodies produced in Chinese hamster ovary cells (Zeitlin et al., 2011). Based on these results, the authors suggest that antibody-dependent cellular cytotoxicity plays a critical role in the protective efficacy conferred by MB-003 (Olinger et al., 2012).

If the treatment with MB-003 began after confirming Ebola infection in rhesus macaques, 43% of animals still survived the challenge (Pettitt et al., 2013). Another monoclonal antibody cocktail, ZMAB, consisting of hybridoma-derived murine neutralizing monoclonal antibodies m1H3, m2G4 and m4G7 (Qu et al., 2011), showed prominent therapeutic efficacy in cynomolgus macaques (Qu et al., 2012). The most advanced, optimized, anti-Ebola monoclonal antibody composition is the ZMapp™ cocktail, consisting of selected components from MB-003 (c13C6) and ZMAb (humanized c2G4 and c4G7), which is produced in ΔXTFT N. benthamiana (Qu et al., 2014), using Magnifexction (Gleba et al., 2005). ZMapp is a product of LeafBio (San Diego, CA), a joint venture of Mapp Biopharmaceutical (San Diego, CA) and Defyrus Inc. (Toronto, ON, Canada), and was manufactured under cGMP at the Kentucky BioProcessing plant facility (http://www.areadevelopment.com/newsItems/10-16-2014/kentucky-bioprocessing-breakthrough-ebola-therapy-owensboro-kentucky-178233.shtml; http://www.mapbio.com; http://www.defyrus.com/products_ebola-zmab.html). Given to rhesus macaques intravenously as three doses 3 days apart, starting as late as 5 days post-EBOV challenge, ZMapp reversed clinical symptoms of advanced EBOV disease (elevated liver enzymes, mucosal haemorrhages and rash) and rescued 100% of treated animals, indicating very promising therapeutic efficacy (Qu et al., 2014).

Although ZMapp safety had not been previously evaluated in humans, it was administered to several Ebola patients as an investigational postexposure therapy during the Ebola outbreak in West Africa in 2014. Of the seven treated patients, five significantly improved and recovered from the disease, despite initiation of the treatment as late as 9 days postinfection (http://edition.cnn.com/2014/08/21/health/ebola-patient-release/index.html; McCarthy, 2014; Lyon et al., 2014). A randomized Phase 1/2 safety and efficacy clinical trial of ZMapp as a putative investigational therapeutic for the treatment of patients with known Ebola infection (by invitation) is currently ongoing (https://clinicaltrials.gov/ct2/show/NCT02363222?term=zmapp&rank=1). Subjects are also being recruited for a Phase 1 open-label trial that will assess safety and pharmacokinetics of ZMapp following a single administration in healthy adult volunteers between the ages of 18 and 50 at a dose of 50 mg/kg (https://clinicaltrials.gov/ct2/show/NCT02389192?term=zmapp&rank=1).

Despite the efficacy of ZMapp in Ebola virus-infected patients, its supply was limited and has been exhausted. Providing the drug in sufficient quantities is a challenge because of the high dose of ZMapp required for optimal therapy (approximately 10.5 g of ZMapp for three administrations to a human being of a 70 kg weight, based on the 50 mg/kg dose given to monkeys [Qu et al., 2014]) and a currently limited capacity for large-scale GMP plant production (Rybicki, 2014). The U.S. Department of Health and Human Services (HHS) has signed a contract with Mapp Biopharmaceutical to accelerate the drug’s development (McCarty, 2014). Several other plant biopharming companies are also gearing up to produce the ZMapp monoclonal antibody components. For example, Medicago (Quebec, Canada), under a task order from the Biomedical Advanced Research and Development Authority (BARDA) at HHS, and as a part of the Indefinite Delivery/Indefinite Quantity (IDI/Q) contract between Medicago and the Defense Advanced Research Projects Agency (DARPA), will be producing three anti-Ebola virus monoclonal antibodies with performance comparable to that of ZMapp, for studying in NHPs. Preliminary results have shown that the technology utilized by Medicago allows for rapidly producing anti-Ebola antibodies with high yields, thereby potentially boosting production amounts and worldwide supply (http://www.medicago.com/files/documents_news/2015/Ebola-PR_USA-_English-Final_v001_j1v036.pdf). Similarly, the Fraunhofer USA Center for Molecular Biotechnology is producing these anti-Ebola virus monoclonal antibodies for BARDA. Additionally, Caliber Biotherapeutics reported on its readiness to produce commercial quantities of ZMapp monoclonal antibodies quickly and cost-effectively (Moore, 2014).

A transient plant expression strategy has also been applied towards the production of an antibody/antigen fusion-type vaccine against Ebola. This recombinant immune fusion complex is based on an anti-Ebola IgG antibody carrying fused GP1 (Phoolcharoen et al., 2011). In a previous study, plant-produced tetanus toxin fragment C–IgG immune complexes were described and reported to bind to the complement factor C1q and Fc
receptor gamma Rila and to be targeted to antigen-presenting cells. These immune complexes, injected subcutaneously without adjuvant, are very highly immunogenic and fully protective in mice, comparable with the commercial antigen adjuvanted with alum. In contrast, plant-produced nonadjuvanted antigen did not induce protective immunity (Chargelegue et al., 2005). To produce Ebola immune complexes, a heavy chain (Hc) and light chain (Lc) of the humanized 6D8 IgG monoclonal antibody directed against a linear epitope on Ebola GP1, with the GP1 subunit genetically fused to the C-terminus of the 6D8 monoclonal antibody Hc, were transiently co-expressed in N. benthamiana plants (Phoolcharoen et al., 2011), using the previously developed bean yellow dwarf virus-based geminivirus replicon system (Huang et al., 2010). Due to the interaction between the 6D8 epitopes and 6D8 binding sites, the recombinant Hc and Lc assembled into a full-size chimeric IgG that formed immune complexes in planta, capable of binding C1q. In mice, subcutaneous immunization with four doses of these purified Ebola immune complexes, without adjuvant, elicited anti-Ebola virus antibody production at levels comparable to those obtained with the positive control, GP1 VLPs (Phoolcharoen et al., 2011), based on recombinant RNA replicons derived from an attenuated strain of Venezuelan equine encephalitis virus (Pushko et al., 2000). In the follow-up study, the authors observed that administration of the Ebola immune complexes along with polynosinic:polycytidylic acid, a Toll-like receptor 3 agonist, as well as adjuvant, induced antigen-specific antibodies and elicited protective immunity against lethal challenge with live mouse-adapted Ebola virus, which correlated with mixed Th1/Th2 humoral responses to the antigen (Phoolcharoen et al., 2011). Overall, the results suggest the strong potential of the Ebola immune complexes as vaccine candidates against Ebola.

Apart from these Ebola immune complexes, there are no reports in the literature on plant-produced vaccine candidates against Ebola, although the Fraunhofer USA Center for Molecular Biotechnology is working on engineering of an Ebola VLP subunit vaccine and its production in N. benthamiana plants.

**Dengue fever**

Dengue virus is a Category A pathogen. The envelope (E) protein of dengue virus mediates host cell receptor binding for viral entry and induces neutralizing antibodies and protective immunity (Modis et al., 2003; Staropoli et al., 1997), which makes the E protein the major target for plant-based dengue subunit vaccine development. Several studies reported on production of E protein-based subunit vaccines against dengue fever. For example, domain III (DIII) of dengue type 2 E protein (D2EII; amino acids 298–400) was transiently expressed in N. benthamiana plants using a TMV-vector-based system, as confirmed by anti-D2EII antibody binding (Saejung et al., 2007). D2EII expression was increased by incorporating the N-terminal plant signal peptide, the C-terminal ER retention sequence and the N-terminal 5’ untranslated region-omega sequence located upstream of D2EII, resulting in high level accumulation of the D2EII protein (0.28% of total soluble protein). Intramuscular immunization of mice with D2EII elicited virus-specific serum IgG with neutralizing activity against dengue type 2 virus. However, the immunogenicity of D2EII was low, even in the presence of the TiterMax Gold adjuvant. This was suggested to be due to the small size of the recombinant antigen (Saejung et al., 2007).

DIII (amino acids 297–394) was also produced in transgenic non-nicotinic tobacco plants under the control of the cauliflower mosaic virus 35S promoter, both as a stand-alone antigen (0.13–0.225% of total soluble protein) (Kim et al., 2009) and as fusion to CTB (0.019% of total soluble protein) (Kim et al., 2010). Despite low expression, DIII-CTB exhibited binding to GM1 ganglioside on intestinal epithelial cells, which is important for its biological activity. Alternatively, DIII was fused to the intestinal M cell-targeting peptide ligand, Co1, to increase its mucosal immunogenicity, and this fusion was expressed in transgenic rice calli under the control of the inducible rice amylase 3D promoter (Kim et al., 2013). The DIII-Co1 fusion protein successfully bound to M cells in Peyer’s patches as confirmed by in vitro and in vivo antigen uptake assays (Kim et al., 2013). No immunogenicity studies of these dengue subunit vaccine candidates were performed.

Another group (Martinez et al., 2010) successfully used the Magnifaction system (Gleba et al., 2005) for transient expression of several versions of the E protein in N. benthamiana plants: the truncated E protein lacking the membrane anchor domain (Et, amino acids 1–397), a polyprotein consisting of Et and the dengue virus structural proteins C and prM (CprMEt, amino acids 1–680) and a D2EIII fusion to HBcAg (HBcAg-DIII, amino acids 289–397). With all these approaches, targets were successfully expressed (approximately 0.4–0.6 mg/g of fresh leaf weight) and reacted with anti-E antibodies, although CprMEt was not fully proteolytically cleaved during expression. Immunogenicity of these recombinant proteins was not reported.

Attempts have also been made to produce a VLP-based dengue subunit vaccine. For example, dengue type 3 virus premembrane and E polyprotein (prME) was expressed in lettuce chloroplasts, and VLP assembly was confirmed by transmission electron microscopy (TEM) (Kanagaraj et al., 2011). Immunogenicity of these VLPs was not reported.

Recently, a recombinant immune complex-based approach, reported previously for Ebola (Phoolcharoen et al., 2011), has been applied towards dengue vaccine development. In a recent study, a hybrid recombinant IgG based on the 6DB anti-Ebola monoclonal antibody was designed, where the chimeric consensuss DIII of the E protein, displaying the Ebola 6D8 epitope, was fused to the C-terminus of the Hc (Kim et al., 2015). The authors transiently expressed these IgG molecules in N. benthamiana plants using a geminivirus vector and demonstrated formation of immune complexes that could bind the complement factor C1q. Mice immunized subcutaneously with these immune complexes with no adjuvant generated potent, virus-neutralizing, DIII-specific serum IgG responses, which were superior to responses observed after immunization with bacteria-produced DIII, also lacking adjuvant (Kim et al., 2015). These results show the potential for the DIII immune complex as a self-adjuvanted dengue vaccine candidate, as well as the universal potential of this platform for vaccine development against other viral infections.

**Yellow fever**

Yellow fever virus is a Category C pathogen. The E protein of yellow fever virus (YFV) plays multiple roles during cell infection (Kaufmann and Rossmann, 2011; Smit et al., 2011; Stiasny and Heinz, 2006) and induces virus-neutralizing antibodies and protective immunity (Pierson and Diamond, 2008; Pierson et al., 2008), which makes it a key target in the development of a subunit vaccine against yellow fever (Després et al., 1988, 1991). Recently, the Fraunhofer USA Center for Molecular Biotechnology has engineered and expressed YFV in N. benthamiana (Tottey, S., 2013).
Crimean–Congo, Rift Valley and Hanta haemorrhagic fevers

Crimean–Congo, Rift Valley and Hanta viruses are Category A pathogens. A recently developed plant-based subunit vaccine candidate against Crimean–Congo haemorrhagic fever (CCHF) comprises a recombinant protein (Gc/Gn) made up of the Gc and Gn portions of the CCHF virus GP (Ghiasi et al., 2011). Gc and Gn have previously been shown to contain immunogenic epitopes capable of inducing virus-neutralizing antibodies (Ahmed et al., 2005). Gc/Gn was expressed in transgenic tobacco hairy root cultures and leaves, to levels of 1.8 mg/kg and 1.4 mg/kg of fresh biomass, respectively. To evaluate immunogenicity of the Gc/Gn subunit vaccine candidate, mice were vaccinated using the following protocols: orally, by administering five 10-μg doses of transgenic leaf or hairy root pellets, at 1-week intervals; orally, by administering four 10-μg doses of transgenic leaf or hairy root pellets at 1-week intervals, followed by a single subcutaneous boost with the amount of total soluble protein equivalent to 5 μg of plant-made Gc/Gn; and subcutaneously, with four doses of an inactivated CCHFV vaccine at 2-week intervals. The unvaccinated mice received a mixed pellet of wild-type leaves and roots. All groups of vaccinated mice developed antigen-specific IgG titres in sera and IgA in faeces, indicative of a mucosal immune response. However, mice that were orally immunized and then subcutaneously boosted demonstrated a significant rise in antigen-specific IgG titres (1:32 000) compared with mice that were only orally immunized (1:256). Plant-produced Gc/Gn reacted with human serum IgG from a patient who had recovered from CCHF. Generally, the results suggest the potential for Gc/Gn as an orally administered vaccine against CCHF; however, for practical administration, low target yields will need to be addressed prior to scaling up production.

In another study, a Rift Valley fever subunit vaccine was designed as either the virus nucleoprotein (N) or a truncated Gn ectodomain and expressed in transgenic Arabidopsis thaliana (Lagerqvist, 2013). Targets were selected based on their potential to stimulate virus-neutralizing antibodies and protection against lethal viral challenge. The N protein accumulated in transgenic plants, whereas expression of truncated Gn was not detectable by western blot. Nevertheless, for both antigens, feeding of mice with the transgenic plant material resulted in elevated serum IgG responses after the second dose. Although N protein-induced IgG titres were higher than those for truncated Gn, they were 10- to 100-fold lower than after a single subcutaneous injection of bacterial derived N protein. Similarly, the nucleocapsid protein of Hantavirus serotype Puumala was produced in transgenic tobacco and potato plants. Transgenic tobacco leaf extract was immunogenic when administered to rabbits intramuscularly or intraperitoneally (Kehm et al., 2001), but transgenic potato tubers or leaf powder did not elicit antibody responses when administered to mice orally (Khattak et al., 2004).

Viral encephalitis

Several types of zoonotic viruses cause encephalitis in humans. For two of them, West Nile and Japanese encephalitis, plant-based therapeutics have been produced and are under development.

West Nile encephalitis

West Nile virus (WNV) is an arthropod-borne flavivirus and Category B pathogen, most commonly transmitted to humans by the bites of infected mosquitoes. WNV can cause febrile illness, encephalitis or meningitis. Transmission of WNV has been documented in Europe, the Middle East, Africa, India, parts of Asia and Australia. In North America, it was first detected in 1999 and has since spread across the continental United States and Canada (http://www.cdc.gov/westnile/faq/genQuestions.html). Currently, there is no vaccine against WNV infection (http://www.cdc.gov/westnile/faq/genQuestions.html).

Domain III (DIII) of WNV E glycoprotein, containing the cell receptor-binding motif and the majority of epitopes inducing strong virus-neutralizing antibodies and protection (Oliphant et al., 2005), is a target of WNV subunit vaccine development. Recently, DIII has been transiently produced in N. benthamiana plants using Magnifection (Gleba et al., 2005), with maximum accumulation detected at 4 days postinfiltration (He et al., 2014b). Of subcellular localizations tested for DIII expression (the cytosol, ER and chloroplasts), target accumulation was highest in the ER (∼73 mg/kg of fresh leaf tissue). The plant-derived DIII was soluble, with correct conformation and epitope display, as demonstrated by specific binding to a monoclonal antibody hE16 recognizing a native WNV DIII conformational epitope. Subcutaneous immunization of mice with four doses of alum-adjuvanted purified DIII elicited potent antigen-specific serum IgG titres that bound to the native form of the viral DIII antigen and were exclusively of the IgG1 isotype, indicating a Th2-type immune response (He et al., 2014b). The importance of IgG1 in the WNV protective immunity has previously been demonstrated by fractionation of WNV-neutralizing human immunoglobulins. The results indicated that IgG1 was the main antibody isotype induced following WNV infection, contained almost the entire in vitro WNV neutralization activity and conferred superior protection on WNV-susceptible mice compared with other isotypes and unfractionated immunoglobulins (Hofmeister et al., 2011). Full-length E protein of WNV was also expressed (Chen, 2015), using glycoengineered N. benthamiana line ΔXTFT, a glycosylation mutant lacking plant-specific N-linked glycan residues (Strasser et al., 2008). Inclusion of domains DI and DII significantly reduced leaf necrosis and increased recombinant protein accumulation to ~600 mg/kg of fresh leaf tissue. Immunization of mice with this full-size E protein, in the presence of alum, elicited robust neutralizing antibody responses, specific for both the E protein and DIII, and induced cytokine release. Both IgG and cytokines were predominantly of the Th2 type (Chen, 2015).

In addition to monomeric protein subunit vaccines, VLP-based subunit vaccines against WNV have also been engineered and produced. Infiltration of N. benthamiana plants with TMV-based MagnICON vectors (Gleba et al., 2004), which encode premembrane M (prM) and E proteins of WNV New York 1999 strain, resulted in transient expression of prM, mature membrane M and E proteins and in their assembly into enveloped VLPs. These VLPs are undergoing immunogenicity testing in mice (Chen, 2015; Chen and Lai, 2013). Chimeric nonenveloped VLPs were also engineered, in which DIII was fused to HBCAg. The HBCAg–DIII fusion antigen was transiently expressed in N. benthamiana plants using either geminiviral vectors or MagnICON vectors, yielding high levels of target accumulation (∼350 mg/kg versus ∼1.0 mg/g of fresh leaf biomass, respectively). These chimeric VLPs were
strongly immunogenic, with a single dose of 25 μg able to elicit potent DIII-specific humoral and cellular immune responses in mice (Chen, 2015; Chen and Lai, 2013; Chen et al., 2011).

An antibody-based therapy may also be a promising approach for the treatment of WNV infection, and several candidates have been produced in plants. Using MagnICON vectors (Giritch et al., 2006) and a geminivirus vector (Hu et al., 2010), the first version of a monoclonal antibody against DIII of WNV E protein, a humanized fully assembled monoclonal antibody hE16, was transiently produced in N. benthamiana and lettuce, respectively (Lai et al., 2010, 2012). High levels of target accumulated in leaf tissues (800 mg/kg and ~260 mg/kg of fresh biomass for N. benthamiana and lettuce, respectively) (Lai et al., 2010, 2012). Plant-produced hE16 retained high-affinity antigen binding and potent virus-neutralizing activity in vitro. Furthermore, a single dose of plant-produced hE16 protected mice against lethal challenge with WNV in both prophylactic and therapeutic settings, even if given as late as 4 days after infection. Protective efficacy of plant-produced hE16 was equivalent to that achieved with mammalian cell-produced hE16 (Lai et al., 2010).

In an effort to improve hE16 monoclonal antibody efficacy, several modified versions of hE16 monoclonal antibody have also been engineered and transiently expressed in wild-type N. benthamiana. These included full-size plant-based hE16, hE16scFv–CH2–3, a single-chain variable fragment (scFv) of hE16 fused to the constant domains of human IgG Hc (CH2), and Tetra-hE16, a tetravalent molecule assembled from hE16scFv–CH2–3 with a second hE16scFv fused to the constant domain of Lc (CH3) (He et al., 2014a,b). Both hE16scFv–CH2 and Tetra-hE16 were efficiently expressed and assembled in plants and were efficacious, exhibiting at least equivalent in vitro neutralization and in vivo mouse protection compared to mammalian cell-produced hE16. The study also demonstrated differences in the N-glycosylation patterns of these hE16 variants, suggesting that proper pairing of Hc and Lc was essential for complete N-glycosylation of the antibodies. Differential glycosylation resulted in different binding of the variants to C1q and specific Fc gamma receptors. None of these variants showed antibody-dependent enhancement activity in vitro, indicating the potential of plants to produce antibodies or antibody-like molecules that minimize this pathogenic reaction in patients that occurs as a result of the Fc–Fc gamma receptor interaction (He et al., 2014a,b).

In another study (Lai et al., 2014), to produce targets carrying mammalian-like N-linked glycans, hE16 and hE16scFv–CH2 were expressed in the glycoengineered N. benthamiana line AXFT (Strasser et al., 2008). The resulting glycoengineered variants exhibited enhanced WNV neutralization in vitro and equivalent protection of mice against a lethal WNV challenge, even at 4 days postinfection, to the parental mouse E16 monoclonal antibody. Between the two glycoengineered variants, expression and protective efficacy were equivalent, indicating the potential of hE16scFv–CH2 as an alternative to hE16 (Lai et al., 2014). Overall, the data demonstrate the feasibility of cost-effectively producing anti-WNV monoclonal antibody-based therapies with an improved safety profile.

Japanese encephalitis

Japanese encephalitis (JE) is a zoonotic infection caused by a Category C flavivirus, observed in Asia and the western Pacific, which can be transmitted to humans via the bite of an infected mosquito. Although most human cases are mild, a small percentage of infected persons develop encephalitis, with an approximately 25% mortality rate (http://www.cdc.gov/japaneseencephalitis/). A vaccine for JE that is licensed in the United States, Ixiaro (manufactured by Intercell, Livingston, UK, and distributed by Novartis Vaccines and Diagnostics, Cambridge, MA), is an inactivated JE virus (JEV) propagated in Vero cell culture and is approved for children over 2 months of age and for adults. Vaccination is recommended for travellers to Asia, as well as for laboratory workers at risk of exposure to JEV (Intercell Biomedical, 2013). Vaccine safety remains to be evaluated in a larger human population (Hills et al., 2013). Similar to WNV, plant-produced E protein-based subunit vaccine candidates have been developed against JEV, using transgenic tobacco (Appaiahgari et al., 2009) and rice (Wang et al., 2009). In contrast to mammalian cells, transgene expression in plants could occur only in the absence of the pRM protein (Appaiahgari et al., 2009). Target expression in leaves of transgenic rice reached 0.11–0.19% of total soluble protein. Protein extract from E protein expressing transgenic rice was shown to be immunogenic in mice, inducing strong E-specific neutralizing IgG titres after intraperitoneal immunization, and both systemic and mucosal antibody responses after oral immunization (Wang et al., 2009).

Influenza

Although influenza virus is only classified as a Category C pathogen, it remains a major public health concern as a reemerging infection. From 1976 to 2007 in the United States, the estimated annual average of influenza-associated deaths, resulting from respiratory and circulatory causes, including pneumonia and influenza, was about 23 600 (ranging from 3349 to 48 614), which corresponds to a range of 1.4–16.7 deaths per 100 000 persons (CDC, 2010). During annual epidemics, 3–5 million cases of severe influenza illness are recorded worldwide, resulting in 250 000–500 000 deaths (www.who.int/mediacentre/factsheets/fs211/en/index.html). In addition to seasonal influenza outbreaks, occasional influenza pandemics can arise at any time when an influenza A virus containing a novel HA subtype emerges and spreads efficiently among humans. In 2009, a novel influenza A H1N1 virus of swine origin [A(H1N1)pdm09] emerged and infected humans. The virus, a triple reassortant with genes acquired from swine, avian and human influenza viruses (Garten et al., 2009), was first detected in people in the United States in April 2009 (Novel Swine-Origin Influenza A (H1N1) Virus Investigation Team et al., 2009) and spread rapidly across the globe, primarily in children and young adult populations with little pre-existing serologic immunity to the novel HA (Hancock et al., 2009), causing the World Health Organization (WHO) to declare a pandemic on 11 June 2009 (http://www.who.int/mediacentre/news/statements/2009/h1n1_pandemic_phase6_20090611/en/). Nearly 30 000 confirmed cases in 74 countries were reported. Of those, about 2% developed severe illness, often with very rapid progression to life-threatening pneumonia, although the number of deaths was small (http://www.who.int/mediacentre/news/statements/2009/h1n1_pandemic_phase6_20090611/en/). Furthermore, a highly pathogenic avian influenza (HPAI) A (H5N1) virus that spread rapidly from 2004 onwards and caused serious poultry outbreaks in several Asian countries, Europe and Africa is also able to infect humans. Among more than 500 individuals who have been infected with H5N1 viruses, the mortality rate is 50–60% (www.cdc.gov/flu/avianflu/index.htm; www.who.int/influenza/human_animal_interface/avian_influenza/en/; http://www.who.
int/influenza/human_animal_interface/HSN1_cumulative_table_archives/en/). Although almost all reported human cases have had close contact with infected birds, with very rare instances of presumed human to human transmission, considerable concern for human health remains, not only because of the severity of human cases, but also because the virus could undergo adaptive mutation or reassortment and obtain the ability to spread efficiently among humans (www.cdc.gov/flu/avianflu/index.htm; www.who.int/influenza/human_animal_interface/avian_influenza/en/; http://www.who.int/influenza/human_animal_interface/HSN1_1_cumulative_table_archives/en/). Should these events occur, HSN1 and/or other novel viruses could have the potential to cause a pandemic exhibiting a very aggressive clinical progression with devastating consequences for global health and economies.

Influenza infection is caused by enveloped, single-stranded, negative-sense RNA orthomyxoviruses of A, B or C type (Lamb and Krug, 2001; Ruigrok, 1998). Traditionally, influenza viruses are manufactured by propagating influenza viruses in embryonated hens' eggs. Because of continued changes in the multiple circulating epidemic strains of influenza viruses and a lack of pre-existing immunity to emerging viruses, manufacturing of new upgraded vaccines is required on an annual basis. HA, the most abundant surface glycoprotein of influenza virus, responsible for binding to cell surface receptors and internalization of the virus, plays a key role in virus infectivity and pathogenesis (Skehel and Wiley, 2000; Wilson et al., 1981). It is well recognized that protection provided by influenza vaccines is mediated primarily by anti-HA highly neutralizing antibodies (Han and Marasco, 2011), making HA the major target for influenza subunit vaccine development (Athmaram et al., 2011; Cox, 2008; Mischler and Metcalfe, 2002; Wu et al., 2010). Anti-HA antibodies can inhibit the hemagglutination activity of HA, and this hemagglutination inhibition (HAI) is used as a correlate of protection for the evaluation and licensing of influenza vaccines (US FDA, 2007).

Traditional influenza vaccines, manufactured using embryonated hens' eggs in the form of inactivated influenza viruses, are generally safe and efficacious against the infection (Matthews, 2006; Osterholm et al., 2012; Schultz-Cherry and Jones, 2010). For example, Fluzone® (Sanofi Pasteur, Swiftwater, PA) is an approved trivalent or quadrivalent split-virus vaccine, containing HAs of influenza A and B strains and reformulated annually for each influenza season, and given in one or two doses (Sanofi Pasteur, 2014a,b). Despite the traditional influenza vaccine benefits, their production is facing a number of challenges associated with dependence on egg supply, potential virus mutations during egg adaptation (Skowronska et al., 2014), propagation of live viruses and egg allergenicity. These challenges would potentially be overwhelming during a pandemic. Progress towards the development and marketing of influenza subunit vaccines has been marked by the recent approval of Flublok® (Protein Sciences, Meriden, CT), a trivalent seasonal influenza vaccine made in the insect cell system (Protein Sciences Corporation, 2014).

Many studies have been reported on the development of plant-based influenza subunit vaccines (reviewed in Chichester et al., 2009a; D’Aoust et al., 2010; López-Macías, 2012; Redkievicz et al., 2014; Yusibov et al., 2015). In regard to structure, three types of plant-produced influenza subunit vaccine candidates have been produced: monomeric HA, trimeric HA and HA-based enveloped VLPs. These plant-based influenza subunit vaccine candidates have been extensively studied in animal models and shown to induce protective immunity (D’Aoust et al., 2008; Landry et al., 2010; Mett et al., 2008; Shoji et al., 2009, 2011, 2013, 2015). Some subunit vaccines, particularly monomeric HAC1 and HAI-05 developed by the Fraunhofer Center for Molecular Biotechnology (Chichester et al., 2012; Cummings et al., 2014) and HA-based VLPs for seasonal (H1N1 and other strains) and pandemic (HSN1 and H7N9 strains) influenza developed by Medicago (Landry et al., 2010, 2014; Ward et al., 2014, www.clinicaltrials.gov; www.medicago.com), progressed to clinical trials, where their immunogenicity and safety, as well as the effect of adjuvants, were evaluated in healthy human volunteers (Table 3) (reviewed in Yusibov et al., 2015).

In addition to HA-based subunit vaccines, which are susceptible to antigenic changes in HA, attempts are being made to engineer plant-based universal influenza subunit vaccines, using influenza virus proteins that evolve more slowly than HA. For example, potato virus X-based chimeric VLPs displaying an H-2Dβ-restricted epitope of nucleoprotein of influenza A virus strain A/PR/8/34 have been designed, transiently produced in N. benthamiana and showed to stimulate MHC class I-restricted peptide-specific IFNγ-secreting CD8+ T cells in mice in the absence of adjuvant (Lico et al., 2009). Engineered CPMV-based VLPs displaying the ectodomain epitope of the M2 protein (M2e) of human or avian influenza A viruses (M2Eh and M2eA, respectively) and transiently expressed in Vigna unguiculata have also been produced (Meshcheryakova et al., 2009). Due to poor yield and weak animal immunogenicity, these chimeric CPMV-M2e VLPs are unlikely to progress to clinical development. The M2e influenza epitope was also engineered as a fusion to the human papillomavirus 16 L1 carrier protein and expressed in N. benthamiana using a CPMV-derived vector (Matic et al., 2011). Immunogenicity and protective efficacy of this vaccine candidate have not been reported.

SARS

Another emerging infection that has raised major concern is SARS, a serious form of pneumonia caused by a SARS coronavirus (SARS-CoV), a Category C pathogen, which was first identified in China in 2003. Within several months, SARS quickly spread around the world, infecting an estimated 8000 people with 750 deaths, and often requiring hospitalization and breathing assistance. The virus is spread through inhalation or touch of sneezed or coughed droplets from an infected person (http://www.ncbi.nlm.nih.gov/pubmedhealth/PMH0004460/; Mclntosh and Perlman, 2009). The WHO identified SARS as a global health threat and issued special biosafety guidelines for handling SARS-CoV specimens and cultures (http://www.who.int/csr/sars/en/). Since 2004, there have not been any reported cases of SARS (http://www.cdc.gov/sars/). Currently, there is no vaccine against SARS (http://www.who.int/immunization/topics/sars/en/).

The spike (S) glycoprotein of CoVs is known to be the major inducer of virus-neutralizing antibodies responsible for protective antiviral immunity (Callebaut et al., 1996; Torres et al., 1995). Furthermore, the truncated version of the SARS-CoV S protein (S1) has been shown to contain important antigenic domains (Babcock et al., 2004). Therefore, both the full-length and truncated versions of the S protein are targets for subunit vaccine development against SARS infection (Yang et al., 2004). There are several reports on the production of S protein-based subunit vaccines in plants. In one study, the 79-kDa N-terminal fragment of SARS-CoV S protein (S1) was expressed in transgenic tomato and low-nicotine tobacco and evaluated as a potential orally administered vaccine (Pogrebnyak et al., 2005).
A single oral administration (4- to 5-h-long feeding) of the lyophilized S1 transgenic tomato fruits, but not intragastric delivery of reconstituted lyophilized transgenic tobacco root material, induced secretion of mucosal IgA in mice detected in faecal pellet extracts. Lower immunogenicity of transgenic tobacco may be due to the lower amount of material administered to mice, smaller dose of antigen and shorter time of mucosal exposure to the antigen compared with transgenic tomato.

The effect of parenteral booster immunization on immunogenicity of the plant-produced S1 protein was also studied. An intraperitoneal boost with the commercial N-terminal S protein peptide, following three intragastric immunizations with S1 transgenic tobacco root material, did not elicit S protein-specific serum IgG responses. In contrast, the same boost following parenteral immunization of mice with S1 transgenic tobacco root material (two times subcutaneously, with Freund’s adjuvant, and one time intraperitoneally, without adjuvant) induced the development of a systemic immune response, eliciting SARS-CoV-specific serum IgG titres predominantly of the IgG1 isotype. The response magnitude was equivalent to the secondary immune response elicited by the commercial S peptide adjuvanted with complete Freund’s adjuvant (Pogrebnjak et al., 2005).

In another preliminary study, a partial S protein of SARS-CoV was produced in transgenic tobacco and lettuce plants, transplasmonic tobacco plants and transiently expressing tobacco plants (Li et al., 2006). However, immunogenicity has not been reported.

A 193-amino acid fragment of SARS-CoV S protein receptor-binding domain (RBD), fused with CTB, was also stably expressed in tobacco chloroplasts, to facilitate mucosal immunity following oral delivery of this vaccine candidate. The CTB–RBD fusion protein was expressed mostly in soluble monomeric form and its highest expression, 10.2% of total soluble protein, was obtained from mature transplastomic leaves (Zhong et al., 2014). Again, immunogenicity has not been reported.

In addition to the S protein, the nucleocapsid (N) protein of SARS-CoV has also been assessed as a potential vaccine target. The molecule has been transiently expressed in N. benthamiana to a level of 79 mg/kg of fresh leaf weight at 3 days postinfiltration (Zheng et al., 2009). Mice injected intraperitoneally with pretreated plant extract emulsified in Freund’s adjuvant developed strong N protein-specific serum IgG titres (~1:1800 following three immunization), including IgG1 and IgG2a isotypes. In addition, IFNγ and IL-10, but not IL-2 and IL-4, were up-regulated in splenocytes at different time points, indicating that the plant-based SARS-CoV N protein induced both humoral and cellular immune responses (Zheng et al., 2009). An alternative adjuvant suitable for use in humans will be needed for further development of this vaccine candidate.

In addition to a vaccine, antiviral compounds may be promising countermeasures against viral infections. For example, griffithsin, the 12.7-kDa lectin from the red algae Griffithsia sp. with an antiviral activity against human immunodeficiency virus (HIV), binds terminal mannose residues of N-linked glycans on the surface of the HIV envelope glycoprotein gp120 and blocks virus entry into host cells (Mori et al., 2005). Due to its structure (the presence of carbohydrate recognition domains and formation of domain-swapped dimers) (Ziolkowska et al., 2006), griffithsin may have a broader activity and inhibit other enveloped viruses that display similar glycans structures. For example, recombinant bacteria-derived griffithsin has been shown to be active against JEV in vitro and in vivo, completely protecting mice against lethal viral challenge (Ishag et al., 2013). The potential of griffithsin is further supported by its successful production in N. benthamiana using TMV-based transient expression approach, with a yield of 300 mg/kg of fresh biomass, and recovered at a purity of >99% (O’Keefe et al., 2009). The plant-produced griffithsin exhibited antiviral activity against a broad spectrum of coronaviruses, including SARS-CoV, via specific binding to the SARS-CoV spike glycoprotein (S protein) (O’Keefe et al., 2009). The efficacy of griffithsin was demonstrated using a lethal mouse model of pulmonary infection, where intranasal treatment with griffithsin protected animals against SARS infection. Mechanisms of CoV disease pathogenesis are believed to involve excessive and dysregulated cytokine/chemokine responses by pro-inflammatory cells (Perlman and Dandekar, 2005). The results of the current study support this hypothesis: treatment with griffithsin significantly reduced the production of several cytokines in mouse lung tissue, including IL-1α, IL-1β, IL-6, G-CSF, MCP-1 and IL-12(p40), by 4 days postinfection, compared results with untreated infected mice (O’Keefe et al., 2010).

Another high-mannose glycan-specific lectin, actinohivin from an actinomycete (Inokoshi et al., 2001), has been produced in N. benthamiana using a TMV-based vector and has been shown to have activity against HIV in a syncytium formation in vitro assay (Matoba et al., 2010). A version of a plant-produced recombinant fusion of actinohivin to the Fc portion of a human monoclonal antibody was recently reported to exhibit nanomolar antiviral activity against HIV as well as other enveloped viruses, including influenza virus, hepatitis C virus, Ebola virus and SARS-CoV, suggesting both clinical and diagnostic applications for this hybrid polypeptide (Matoba, 2014).

Toxin poisoning

Several toxins of bacterial or plant origin are also considered bioterror agents (Table 1). These toxins can be easily isolated and spread with food or water or as aerosols, posing major threats to human health.

**Botulinum**

Botulinum neurotoxin, the most lethal naturally occurring toxin and a Category A agent, is produced by the anaerobic bacterium *Clostridium botulinum*. It causes botulism, a rare but serious and life-threatening food-borne disease (www.niaid.nih.gov/topics/botulism/, http://www.who.int/mediacentre/factsheets/fs270/en/, Arnon et al., 2001; Montecucco and Molgó, 2005). Treatment of botulism includes administration of an antitoxin as soon as possible after establishing a clinical diagnosis (www.niaid.nih.gov/topics-botulism/, Arnon et al., 2001; Montecucco and Molgó, 2005). In 2013, the U.S. FDA approved the use of an antitoxin that neutralizes all seven known botulinum neurotoxin serotypes (US FDA, 2013). A vaccine against botulism, investigational pentavalent (ABCDE) botulinum toxoid vaccine for workers at risk for occupational exposure to botulinum toxins, was developed several decades ago and, due to decreased immunogenicity and increased reactogenicity of the vaccine stock, was discontinued in 2011 (CDC, 2011).

In an attempt to develop a plant-based subunit vaccine against botulism, MucoRice, a rice-based expression system for the production of mucosal vaccines (Nochi et al., 2007; Yuki et al., 2009), was combined with RNAi technology, to suppress accumulation of major rice endogenous storage proteins, to produce a botulism vaccine antigen in rice seed. Using this
system, the 45-kDa C-terminal half of the Hc of botulinum type A neurotoxin (BoHc) was expressed in rice at an average of 0.1 mg per seed (Yuki et al., 2012). The target, MucoRice-BoHc, is water soluble and accumulated in the cytoplasm but not in protein bodies I or II of rice seeds. Mucosal (intranasal) immunization of mice with MucoRice-BoHc conferred protection against a lethal challenge with botulinum type A neurotoxin. When MucoRice-BoHc was intranasally administered in the presence of CT or a nontoxic chimeric adjuvant mCTALLTB (a combination of the A subunit of mutant CT E112K and the pentameric B subunit of heat-labile enterotoxin from enterotoxigenic E. coli), high levels of antigen-specific serum IgG and nasal IgA responses were induced and all the immunized mice survived the challenge (Yuki et al., 2012).

An antibotulinum toxin A-neutralizing scFv recombinant monoclonal antibody has also been produced in plants. The functional activity of the antibody produced in transgenic tobacco was demonstrated in vitro using mouse muscle twitch assays (Almquist et al., 2006). Based on the efficiency of the scFv capture process and the dose required to provide an antidote to a human, the authors calculated that 1–2 ha of transgenic tobacco could yield up to 4 kg of scFv, which would allow for the production of 1 000 000 therapeutic doses (10 mg of each botulinum toxin A-specific antibody as an oligoclonal cocktail for an average 70 kg person with a blood volume of 5 L) (Almquist et al., 2006), indicating the potential of this production system for antibotulinum scFv manufacturing.

**Ricin**

Ricin, the toxin from *Ricinus communis* (castor bean plants), is a Category B agent. Ricin is easy to extract, very stable and highly toxic with rapid and irreversible action. Symptoms of ricin poisoning and time of death depend on the route of exposure (inhalation, ingestion or injection) and the dose received, but the toxin affects many organs. Ricin poisoning is symptomatically treated by supportive medical care (http://www.bt.cdc.gov/agent/ricin/facts.asp; Balali-Mood et al., 2013). A recombinant thermostable vaccine candidate against ricin, RiVax™ (Soligenix, Princeton, NJ), is currently under development. In mice, intramuscular immunization with RiVax protected against challenge with ricin delivered by gavage or inhalation (Smallshaw et al., 2007). In the NHP model, RiVax conferred 100% protection against lethal inhalational challenge with aerosolized ricin (Roy et al., 2015).

Additional countermeasures against ricin intoxication, which are under development, are based on a monoclonal antibody GD12 recognizing an immunodominant linear neutralizing epitope (amino acids 163–174 [TLARSFLICIQM]) on the A subunit of ricin toxin and demonstrating efficacy both in vitro and in vivo (Neal et al., 2010). In vitro, using a Vero cell-based cytotoxicity assay, murine hybridoma-produced GD12 was shown to bind ricin holotoxin with high affinity (dissociation constant of 2.9 × 10^-19 M) and neutralize it with a 50% inhibitory concentration of ~0.25 μg/mL. In vivo, passive administration of GD12, by intraperitoneal injection or via the ‘backpack tumour’, protected mice against systemic (intraperitoneal) and mucosal (intragastric) ricin challenge, respectively (Neal et al., 2010). Based on the properties of the murine GD12 monoclonal antibody, an engineered version of this monoclonal antibody was produced in plants. The plant-derived version was a chimeric derivative of GD12 (cGD12), in which the murine Hc and Lc variable regions were fused to a human IgG1 framework, which was engineered and transiently expressed in *N. benthamiana* (O’Hara et al., 2012). Plant-produced cGD12 demonstrated epitope specificity and ricin neutralizing activity similar to those of the native murine monoclonal antibody. Passive administration of cGD12 by intraperitoneal injection protected mice against a systemic ricin challenge. To assess the efficacy of this monoclonal antibody postricin exposure, GD12 and cGD12 were administered 6 h after toxin challenge and shown to rescue mice from toxin-induced death. Thus, plant-produced cGD12 has potential as both a prophylactic and a therapeutic treatment against ricin intoxication.

**Conclusions**

Recent emergence and reemergence of infectious diseases as well as the risk of bioterror attacks pose a major threat to public health and challenge the effectiveness of current prophylactic and therapeutic countermeasures. Whereas vaccines are the mainstay of infectious disease prophylaxis, traditional vaccines are failing to satisfy the global demand because of limited scalability of production systems, long production timelines and product safety concerns. Similar issues are applicable to therapeutic antibodies and anti-infective agents. These concerns may be addressed by the development of safe, cost-effective and scalable plant-based subunit vaccines, monoclonal antibodies and other recombinant therapeutic proteins.

Over the past decade, subunit vaccines, monoclonal antibodies and antivirals against a range of emerging, reemerging and bioterrorism-related pathogens have been produced in different plant expression systems and evaluated in preclinical and clinical studies. In recent years, there have been marked improvements in the levels of plant-produced target expression, yield, purity and reproducibility and in the construction of facilities capable of producing material under cGMP for clinical trials and commercial use. Improved yields and efficacy of these candidate countermeasures suggest feasibility of the use of plant-based products in the fight against such diseases.

However, the difference between the number of preclinical studies (summarized in Table 2) and clinical trials (summarized in Table 3) conducted on these plant-produced candidate countermeasures is considerable. This partly reflects the normal narrowing down of potential product candidates from preclinical to clinical studies, but also reflects the fact that many of the preclinical studies were proof-of-principle investigations, not necessarily targeted for the clinic. However, there is increasing momentum to advance lead plant-based candidates into the clinic, and in recent years, the regulatory and commercial landscape has started changing in favour of plant-derived subunit vaccines, monoclonal antibodies and other therapeutic proteins for human use, exemplified by the licensing and commercial release of taliglucerase alfa. However, to approve and commercialize a plant-produced candidate, regulatory authorities consider not only its safety and efficacy, but also its additional factors, including the effective dose, the number of required doses and product stability during storage. In addition, while some subunit vaccines require an adjuvant, others, such as virus-like particles, may be efficacious alone. These factors would reflect the ultimate product cost. With regard to product stability, subunit vaccines or therapeutic proteins delivered via oral route may undergo rapid degradation in the gastrointestinal tract, which stresses the need for protective formulation.

In addition, to achieve more rapid clinical development of plant-based countermeasures, downstream processing and
protein purification approaches need to be further developed, for manufacturing of targets at large scale. Although a few medium- and large-scale plant-based facilities have been built in the North America and Europe, considerable validation and qualification efforts need to be undertaken to bring these online for commercial production.

Conflict of interest

The authors have no conflict of interest to declare.

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