

Chapter 10

Rapid and Low-Cost Tools Derived from Plants to Face Emerging/Re-emerging Infectious Diseases and Bioterrorism Agents



Rosella Franconi, Elena Illiano, Francesca Paolini, Silvia Massa, Aldo Venuti, and Olivia Costantina Demurtas

Abstract Whether naturally occurring or man-made, biological threats pose a severe risk in an increasingly globalized world.

The dual-use nature of biological research, with its most recent advances in biotechnology ('synthetic biology', gene editing, nanotechnologies etc.) and the rapid diffusion of knowledge, raise proliferation concerns of biological weapons by non-state actors.

Thus, there is an urgent need to develop measures intended to enhance diagnostic, prophylactic and therapeutic capabilities and capacities to improve the ability of society to combat infectious diseases outbreaks, as well as to alleviate the effects of bioterrorism attacks.

We present here two examples of biotechnology usage for biodefence purposes: (i) plants as biofactories for the rapid production of improved biopharmaceuticals ('Plant Molecular Farming'), and (ii) plant sequences as immune-modulating agents to enhance the efficacy of genetic vaccines.

These platforms represent two promising (and complementary) approaches for the rapid and low-cost production of countermeasures (diagnostics and vaccine candidates) against emerging, re-emerging and bioterrorism-related infections.

R. Franconi (✉) · E. Illiano · S. Massa · O. C. Demurtas
Department for Sustainability, Italian National Agency for New Technologies, Energy and the Environment (ENEA), 'Casaccia' Research Centre, Rome, Italy
e-mail: rosella.franconi@enea.it

F. Paolini · A. Venuti
HPV-UNIT – RIDAIT Department, UOSD Tumor Immunology and Immunotherapy, Regina Elena National Cancer Institute, Rome, Italy

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10.1 Introduction

10.1.1 *Biological Threats: Natural Infections and Biological Weapons*

Infectious diseases represent a significant burden on public health and economic stability of societies all over the world being the cause of approximately 25% of 60 million of deaths (in developing countries this percentage reaches 45% of deaths).¹ In 2015, about 50% of all deaths among children under 5 years of age were due to infectious diseases.²

In a globalized world (more travels, trade and greater interconnectedness between countries) infectious disease outbreaks are becoming inevitable, and they remain unpredictable. When faced with diseases for which there are few or no medical countermeasures, massive chaos and considerable loss of lives can ensue: countries without adequate health services are the most vulnerable to the impacts of infectious diseases due to the difficulty in administering effective medical treatments at an early stage and putting effective preventative measures into place.

The spectrum of the biological risk represented by infectious diseases is continuous, including events that can be difficult to distinguish as natural (i.e. natural occurring pandemics, re-emerging infectious diseases, unintended consequences of research), accidental (due to laboratory accidents, ignorance or negligence) or intentional (due to sabotage or biowarfare). Whatever the origin is, ‘*a health threat anywhere is a health threat everywhere*’.³ Thus, it is necessary to identify emerging epidemics as soon as possible, stop them before they spiral out of control and develop suitable medical countermeasures, such as novel and effective vaccines.

10.1.1.1 Natural Infections

Infectious diseases have been for centuries among the leading causes of death and changed the fate of entire civilizations [1]. ‘Black Death’ (plague) in the Middle Ages (1348–1350) killed 30–60% of Europe’s population; smallpox, in the twentieth century, was responsible for 300–500 million deaths and decimated and

¹ World Health Organization, WHO, “The top 10 causes of death” January 2017 <http://www.who.int/mediacentre/factsheets/fs310/en/index2.html>).

² WHO, “Child mortality and causes of death”, http://www.who.int/gho/child_health/mortality/causes/en/).

³ <https://publichealth.wustl.edu/a-health-threat-anywhere-is-a-health-threat-everywhere/>

weakened native populations in the Americas and Australia in the eighteenth century, prior to its final eradication in the late 1970s; measles for centuries caused massive destruction to native populations especially in the Americas and Europe over the years: in 2000 it was declared eradicated in the US but, in spite of the availability of a safe and cost-effective vaccine, it continues to circulate in various parts of the world, causing many deaths globally particularly among young children.⁴ Spanish Influenza epidemic (1918–1919) killed as many as 40 million people worldwide and it considered the most devastating epidemic recorded in world history and a global disaster [2].

Over the past decades, at least 30 novel infectious agents affecting humans have emerged, most of which are zoonotic and whose origins have been shown to correlate significantly with environmental/ecological (i.e. climate change, floods, change of agricultural practices, natural disasters, habitats destruction) and socioeconomic (i.e. increase in population density, falling living standards, decline of infrastructures, human travels, conflicts and social instability, killing of wild animals for food) factors. These factors, together with the natural evolution of pathogens, are constantly leading to facilitate infections in humans, changing the nature of biological risks and increasing the global impact [3]. In particular, *newly emerging* infections refer to diseases that have been discovered in the human host for the first time (i.e. the severe acute respiratory syndrome –SARS- coronavirus, SARS-CoV) while *reemerging infectious diseases* can be defined as infectious diseases that reappear, usually in more pathogenic form and in rapidly increasing incidence or new geographic locations after apparent control or eradication (i.e. Filoviruses like Ebola and Marburg).⁵

The field of emerging disease exploration has been strengthened by the creation of dedicated emerging diseases units and programmes at the Centre for Disease Control and Prevention (CDC)⁶ or at the European Centre for Disease Prevention and Control (ECDC).⁷ These institutions monitor current infectious disease outbreaks, assess the risk to public health and provide technical support to the US/EU level-response to such threats.

10.1.1.2 Bioweapons

The emergence of some pathogens can be the result of deliberate human action, being employed as biological weapons (or ‘bioweapons’, ‘biological warfare’, BW) for destruction. Among the so-called ‘CBRN’⁸ weapons, biological weapons include

⁴ WHO, “Measles,” <http://www.who.int/mediacentre/factsheets/fs286/en/>

⁵ WHO, “Emerging diseases,” http://www.who.int/topics/emerging_diseases/en/

⁶ WHO, “Global infectious disease surveillance,” <http://www.who.int/mediacentre/factsheets/fs200/en/>

⁷ <https://ecdc.europa.eu/en/about-us/who-we-are/disease-programmes/emerging-and-vector-borne-diseases-programme>

⁸ CBRN: chemical, biological, radiological and nuclear.

deadly pathogens – bacteria or viruses – or toxins that can be deliberately released in order to cause harm to people or animals and plants ('agroterrorism'). In addition to potentially catastrophic immediate impact, these agents could also trigger long-term disasters, causing regional instability and challenging international security.⁹

Biological agents can be easily grown and disseminated through inhalation, ingestion or skin absorption. Some of them might affect large numbers of people (such as the highly contagious SARS-CoV), while others might be less contagious but more deadly for those they affect (such as Ebola). Since bioweapons use could resemble natural pandemics, it would be very difficult to differentiate between naturally occurring infections and those resulting from malicious use.

In spite of the difficulties in the evaluation of BW true frequency of use and impact in the past, (due to lack of data, manipulation/secret by political authorities etc.), historical analysis has shown that biological agents have been used in several occasions from ancient times through to the twenty-first century to cause panic and terror among civil populations (for a comprehensive history of biological warfare see Barras and Greub [4]).

The Biological Weapons Convention (BWC, signed in 1972 and entered into force in 1975)¹⁰ bestows a prohibition on the weaponisation of biological pathogens and agents.¹¹ In particular, BWC prohibits: (i) the possession of biological agents except for 'prophylactic, protective, or other peaceful purposes'; (ii) the development of technologies intended for the dispersal of biological agents for offensive military purposes; and (iii) the destruction of existing stocks.¹²

Despite the destructive potential of bioweapons and the relative ease with which malicious actors could obtain many of the materials and know-how required to build them, relatively few cases of bioterrorism or sabotage have been recorded in the twentieth and twenty-first centuries: in the period 1970–2014, of a global total of 143 CBRN attacks, 35 used BW.¹³ A potential disincentive for the acquisition and use of BW might be represented by the fact that biological agents are indiscriminate, and cannot be easily contained once released. On the other hand, some specialists have raised the question whether bioterrorism is a myth or reality [5]

⁹ Use of Chemical, Biological, Radiological and Nuclear Weapons by Non-State Actors. Emerging trends and risk factors. Lloyd's Emerging Risk Report – 2016, Chatman House, The Royal Institute of International Affairs, p 31. <https://www.lloyds.com/news-and-insight/risk-insight/library/society-and-security/cbrn>

¹⁰ The BWC currently has 178 states-parties and six signatories (Central African Republic, Egypt, Haiti, Somalia, Syria, and Tanzania). Twelve states have neither signed nor ratified the BWC (Chad, Comoros, Djibouti, Eritrea, Israel, Kiribati, Micronesia, Namibia, Niue, Samoa, South Sudan and Tuvalu).

¹¹ An analogous Chemical Weapons Convention (CWC, signed in 1993 and entered into force in 1997) incorporates a general clause prohibiting the weaponisation of all chemicals. After dedicated UN negotiations, the Treaty on the Prohibition of Nuclear Weapons (TPNW) has been signed by 122 countries only recently (July 7th 2017, <http://undocs.org/A/CONF.229/2017/8>).

¹² <https://www.armscontrol.org/factsheets/bwcsig>

¹³ National Consortium for the Study of Terrorism and Responses to Terrorism (START), 2015. Global Terrorism Database [online]. Available at: <http://www.start.umd.edu/gtd/>

while some others consider BW as a ‘*common aspect of the human behavioural repertoire*’ [6].

As knowledge diffuses rapidly to different parts of the world, through the globalisation of information and communications technology, a growing concern emerges that biologic agents could be easily used as weapons by non-state actors (such as terrorist organisations, saboteurs or lone actors) in the future. Moreover, certain emerging technologies and scientific advances of biotechnology (i.e. nanotechnology, synthetic biology, gene editing) are altering the risk landscape for bio-weapons use in a variety of ways (‘*dual use*’ concept). The malicious use of synthetic or edited pathogens could possibly enable hostile actors to develop weapons that are cheaper, more powerful and easier to use (i.e. deadly viruses such as polio and Ebola can be synthesized using public databases and available technology).

Being highly unlikely that societies could ever completely eliminate vulnerability to biological agents, there are no doubts that the topic of biological warfare poses very difficult problems, opening some novel challenges in the ethical domain [7].

10.1.1.3 Countermeasures and Novel Approaches to face Biothreats

Since biological agents might be more lethal than chemical weapons, more difficult to detect than nuclear weapons and less expensive to be produced using the common technologies available in any biological laboratory, measures intended to enhance mitigation (diagnostic, surveillance, etc.) and adaptation (new therapeutics, vaccines, etc.) capabilities and capacities, alongside with training and education, will improve the ability of society to combat ‘regular’ infectious diseases outbreaks, as well as counteracting the effects of bioterrorist attacks, enhancing society’s resilience.

Novel technologies (such as nanotechnology, new detection technologies, next generation sequencing) could be useful for clean-up and detection, preserving the health and well-being of first responders or assisting local law enforcement in identifying the nature of an outbreak/attack and the kinds of biological agents involved, making responding easier, reducing the destructive and disruptive capacity of biological threats. On the other hands, antimicrobials and vaccines offer possible means for protection toward sudden emerging infectious diseases outbreaks, both natural and intentional. For these, it is important to rely on strategic reserves of therapeutics/vaccines against known biothreat agents as well as having tools/platforms for the rapid production of effective countermeasures against (novel) pathogens.

In general, the pharmaceutical industry is not much involved in vaccines and rarely invests in research and development for diseases with limited market incentive: when the Ebola outbreak began in 2014, vaccine candidates were unavailable because they had stalled in the pipeline. Repeated outbreaks (most recently, Ebola and Zika) have forged a global consensus that current models for developing vaccines for sporadic epidemic are not working, and that a new system is urgently

needed, also in the light that '*Pathogens are not only terrifying, they're expensive*'¹⁴ (the 2003 SARS epidemic cost \$30 billion in only 4 months). Thus, novel global approaches are needed to drive product innovation to prevent and contain future infectious diseases epidemics.

A few years ago, the Defense Advanced Research Projects Agency (DARPA, US) started supporting new technologies that radically accelerate the manufacturing of protein vaccines and protein-based therapeutics. In 2007, after realizing that low-cost, plant-derived vaccines are better tools to control many infectious diseases in humans, DARPA financed projects for the development of cGMP facilities for plant-made vaccines. In 2015, DARPA funded Inovio Pharmaceuticals, Inc. with \$45 million to develop multiple treatment and prevention approaches against Ebola (a DNA-based vaccine against Ebola, a therapeutic DNA-based monoclonal antibody product and a conventional monoclonal antibody to treat Ebola). More recently, the Biological Technologies Office (BTO) of DARPA sponsored the 'Pandemic Prevention Platform' (P3)¹⁵ program whose goal is to achieve an integrated capability that can deliver pandemic prevention countermeasures to patients within 60 days of an outbreak, changing outbreak response by enabling rapid discovery, characterization, production, and testing of efficacious medical countermeasures (i.e. generation of virus stock, including viral unknowns; rapid evolution of antibody candidates; gene-encoded antibody delivery methods).

Another initiative is represented by the Global Health Security Agenda¹⁶ (GHSa), launched in February 2014, a growing partnership of over 50 nations, international organizations, and non-governmental stakeholders. It pursues a multilateral and multi-sectoral approach to strengthen both the global capacity and nations' capacity to prevent, detect, and respond to infectious diseases threats whether naturally occurring, deliberate, or accidental. The idea is that, this capacity, once established, would mitigate the devastating effects of threats posed by highly pathogenic infectious diseases and bioterrorism events by rapidly detecting and transparently reporting outbreaks when they occur, and employing an interconnected global network that can respond effectively to limit the spread of infectious disease outbreaks in humans and animals, mitigate human suffering and the loss of human life, and reduce economic impact.

Besides the so-called "One Health Initiative" (strategies to control diseases across species),¹⁷ more recently (January 2017) the Coalition for Epidemic Preparedness Innovations (CEPI)¹⁸ was launched at the World Economic Forum. It is a partnership of public, private, philanthropic and civil organizations to accelerate (safe and affordable) vaccine development for emerging infectious diseases,

¹⁴<https://pandorareport.org/2017/06/30/pandora-report-6-30-2017/>

¹⁵<https://globalbiodefense.com/2017/04/18/dstl-darpa-intercept-evolving-countermeasures-bioterrorism/>

¹⁶<https://www.ghsagenda.org/>

¹⁷<http://www.onehealthinitiative.com/>

¹⁸<http://www.who.int/medicines/ebola-treatment/TheCoalitionEpidemicPreparednessInnovations-an-overview.pdf>

particularly for diseases that lack market incentives, readying pandemic defences during peacetime. It is based on a memorandum of understanding with the World Health Organization, (WHO), and has established a partnership with the Bill & Melinda Gates Foundation, governments (like India, Germany, Japan, Norway), industry partners and private funders (i.e. Wellcome Trust), academic institutions and civil society organisations among others. According to the WHO 'R&D Blueprint for Action to Prevent Epidemics' (that indicates the priority pathogens against which the development of medical countermeasures are urgently needed)¹⁹ and based on specific criteria (such as risk of an outbreak occurring, transmissibility of the pathogen, burden of disease, feasibility of vaccine development and the current pipeline candidates), as a first step, three diseases were selected (Lassa fever, Nipah virus, and Middle East respiratory syndrome coronavirus, MERS-CoV) to move new vaccines from preclinical to proof of principle studies in humans. However, since there always will be an unknown or a not selected pathogen that it will not be possible to predict, CEPI aims also to support the development of rapid and adaptable vaccine technology platforms, where antigens from a new pathogen can substitute or be added to an existing vaccine.

10.2 Novel Platforms for Vaccine Production

Traditional vaccines against infectious diseases are failing to satisfy the global demand because of limited scalability of production systems and long production timelines (similar issues are applicable to other anti-infective agents). This is especially a problem for emerging pathogens that carry the inherent risk of pandemic spread in a naïve population.

A considerable number of different platform technologies are under development and, among these, plant-derived vaccines and plasmid-based DNA vaccines are encouraging tools.

Plants have emerged as promising platforms for the production of subunit vaccines, monoclonal antibodies and other recombinant therapeutic proteins ('Plant Molecular Farming') due to time and cost efficiency, scalability, lack of harboured mammalian pathogens and ability to perform eukaryotic post-translational protein modification.

The recent apparent success in fighting Ebola virus with plant-made human antibodies put a spotlight on the enormous potential of this platform for applications in human health [8]. So far, several candidate countermeasures against emerging, re-emerging and bioterrorism-related infections have been produced in plants (reviewed in Rybicki [9]; Streatfield et al. [10]). The modularity of molecular engineering provides fast and scalable systems to be used in response to new outbreaks of highly infectious diseases with pandemic potential, such as influenza, malaria, and SARS [11].

¹⁹<http://www.who.int/blueprint/priority-diseases/en/>

In addition, genetic vaccines represent another advantageous platform for the rapid development of novel vaccines to face deliberate or naturally occurring outbreaks due to ease of preparation and general stability at room temperature. In this case plants might be exploited as a source of immune-modulating sequences able to increase the ‘visibility’ to the immune system of weak antigens for the construction of more powerful genetic vaccines.

10.2.1 Plants as Biofactories for the Production of Biopharmaceuticals (‘Plant Molecular Farming’, PMF)

Herbal medicine has formed the basis of health care throughout worldwide since the dawn of civilization, having been extensively utilised by ancient civilisations [12]. Thousands of plant species contributed to the development of important therapeutic drugs used in modern medicine: almost 50% of the synthetic medicines derive from phytochemicals and almost 30% of all pharmaceuticals approved by the US Food and Drug Administration (FDA) have a botanical origin (digoxin, morphine, salbutamol and aspirin represent some successful examples).

The use of plants as bioreactors (‘Plant Molecular Farming’, PMF) is a relatively new bioscience.²⁰ So far, a variety of subunit vaccines, monoclonal antibodies and therapeutic proteins have been produced in plants and other ‘green’ systems [13, 14] including candidate countermeasures against emerging, re-emerging and bioterrorism-related infections [10].

Plants represent ideal platforms for recombinant protein production for several reasons. Lower manufacturing costs have been widely assumed as an intrinsic advantage of plant-based production platforms. Biologic production in plants does not require capital-prohibitive facilities, bioreactors, and expensive culture media but can be easily scaled in relatively inexpensive greenhouses with simple mineral solutions. Plants compete with other expression systems for reduced risks of contamination with human/animal pathogens and ability to perform eukaryotic post-translational protein modification, such as glycosylation. There are differences in N-glycan and O-glycan structures between plants and mammals [15, 16]; nevertheless, the possibility to control the glycosylation pattern (‘glyco-engineering’ or plant ‘glyco-biotechnology’) provides a method for producing proteins with unique and uniform mammalian post-translational modifications, resulting in biologics with increased efficacy with respect to their mammalian cell-produced counterparts (‘bio-betters’) [17].

²⁰It officially entered in the plant science field in the year 2000 as a specific session at the 6th Congress in Plant Molecular Biology, Quebec, Canada.

Recombinant proteins can be selectively expressed in particular plant cell compartments (chloroplast, apoplast etc.) or organs (seeds, roots, tubers etc.), where they are more stable and do not interfere with vegetative growth. Plant cells/tissues/organs can be lyophilized and stored at ambient temperature for many years, maintaining activity of expressed protein drugs. A promising approach is the use of edible plant tissues/organs expressing biopharmaceuticals for direct oral delivery, with no need for exhaustive purification, thus eliminating expensive downstream purification, cold storage and transportation costs [18]. This could be particularly useful for veterinary vaccines against major zoonotic diseases [19].

Furthermore, plant-based expression platforms offer safe, inexpensive and potentially limitless ways to produce therapeutics in a quick and flexible manner. If time and expression level might represent a limit of the transgenic technology, it can be overcome by transient expression mediated by plant viruses or by agroinfiltration [20]. Recently, novel transient expression vectors have been developed that allow the production of vaccines and therapeutics at unprecedented speed [21]. The recent apparent success²¹ in fighting Ebola outbreak of 2014–2016 with a plant-made drug (ZMapp™, a cocktail of three human monoclonal antibodies) brought renewed attention to the field of plant-made biologics for human health whose potential and capacity to produce ‘rapid response’ vaccines had been already demonstrated by the commitment of several US companies in the production of 100 million doses of influenza vaccine a month by using such technology [9].

Another field in which plants could represent ideal production systems is that of antigen preparation for the development of diagnostic test that is particularly useful when a pathogen cannot be grown in the lab or is highly virulent and needs a methodology for safe, fast and affordable production or when it is necessary to rely on ‘high quality’ reagents.

Our early efforts in the field of PMF were focused in the expression of intracellular antibodies (‘intrabodies’) to obtain plants resistant to viral infection (‘plantibody’-mediated resistance). In particular, we dealt with the Cucumber Mosaic Virus, CMV [22] and the Tomato Spotted Wilt Virus (TSWV, family *Bunyaviridae*) that, since the introduction of the vector *Frankliniella occidentalis* in Europe, become one of the limiting factors and one of the most serious threats to vegetable crops in the Mediterranean basin [23].

Later on, we focused on the use of plant-based platforms for the production of recombinant proteins for the development of novel protection/therapy tools and diagnostics to be quickly manufactured, at low cost and with minimal risk against infective agents like the human papillomavirus (HPV) [24–28] or the severe acute respiratory syndrome (SARS) coronavirus, SARS-CoV [29].

²¹The conditions of two infected American health aid workers dramatically improved soon after receiving the plant-derived experimental drug.

10.2.1.1 Case Study 1: Plant Derived SARS-CoV Antigens as Tools for Preventive Vaccines and Diagnostics

Severe acute respiratory syndrome (SARS) emerged in 2002, spreading to 29 countries over 5 continents, leading to more than 8000 infected patients globally²² with a fatality rate of 9.6%. The aetiological agent of the syndrome, rapidly identified as a coronavirus (SARS-CoV), crossed the species barrier to infect humans, showing high morbidity and mortality rates. The end of the SARS outbreak was declared by WHO in July 2003. However, several local outbreaks were subsequently reported in China as a consequence of accidental laboratory contaminations or infections after contact with animals infected with SARS-CoV strains significantly different from those predominating in the 2002–2003 outbreak [30].

For its high transmissibility, high lethality and significant impact on the public health system, SARS-CoV has been defined a class C biological weapon (Centers for Disease Control and Prevention [31]). Currently, there are no approved antiviral treatments for SARS-CoV. Since a SARS epidemic may recur at any time in the future, it has been included in the WHO 'R&D Blueprint for Action to Prevent Epidemics' list and multiple therapeutic approaches against SARS-CoV (and MERS-CoV) are currently under development [32]. A recent example of such efforts is represented by the nucleotide prodrug GS-5734 (currently in clinical development for treatment of Ebola virus disease) that showed inhibition of SARS-CoV and MERS-CoV replication in multiple *in vitro* systems and in a mouse model of SARS-CoV pathogenesis [33]. Another important key to prevent and control a future outbreak of SARS is to develop novel rapid and specific diagnostic methods, in addition to those already available²³ so that suspected patients can be correctly triaged and isolated.

SARS-CoV has four major viral structural components, the spike (S), envelope (E), membrane (M) and nucleocapsid (N) proteins and 16 non-structural proteins [34].

The structural N and M proteins are the most abundant proteins, respectively, in the virus core and in the viral envelope. The N protein, expressed at early stages of infection, triggers an early, powerful antibody response by the host, thus it is considered the best diagnostic target [35]. Furthermore, since the N protein is able to induce a long-term cell-mediated immune response in animal models, it represents a potential vaccine candidate. The production of recombinant N protein has been achieved in a variety of heterologous expression systems, with eukaryotic platforms (such as insect cells, yeast) allowing more efficient and specific diagnostic tests [36].

The membrane M glycoprotein is functionally involved in the assembly and budding of virions from the cell. The M protein contains T cell epitopes [37] and the availability of recombinant M protein, in combination with other recombinant viral

²²WHO Library Cataloguing in Publication Data (2006). SARS: how a global epidemic was stopped ISBN 92 9061 213 4.

²³WHO, Severe Acute Respiratory Syndrome (SARS): Laboratory diagnostic tests, 2003) <http://www.who.int/csr/sars/diagnostictests/en/>

proteins might overcome the concern about the sensitivity and the specificity of N protein-based assay [38–40], thus providing high quality reagents to detect antibodies in the infected human host.

Recently, we demonstrated that plant transient expression systems can be used to produce SARS-CoV N and M antigens [29]. The N and M full-length genes, derived from the Frankfurt I isolate of human SARS-CoV [41], were inserted into different plant expression vectors.

The N protein was expressed in *Nicotiana benthamiana* plants using Potato Virus X (PVX)-mediated infection. The protein was obtained in systemic leaves of 100% infected plants. Differently from the N protein produced in bacteria, the plant-produced N protein doesn't display any proteolysis, demonstrating the suitability of the plant platform for the production of recombinant SARS-CoV antigens (Fig. 10.1).

In addition, we demonstrated that both crude extracts containing N protein, or purified plant-produced N protein, were specifically recognized in immunoblotting by sera derived from Chinese SARS convalescent patients of the 2003 outbreak, and not from patients affected by unrelated respiratory diseases. This study represents the first demonstration that the plant-derived N protein is able to reveal, by direct serology, human N-specific antibodies present in sera of SARS patients, thus pro-

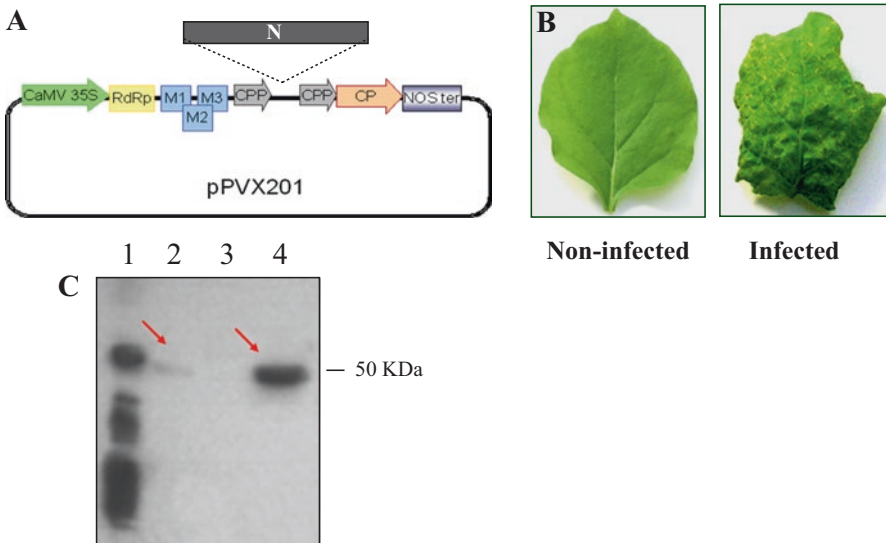


Fig. 10.1 Transient expression of the SARS-CoV N antigen in *Nicotiana benthamiana* leaves. (a) The N gene was inserted into a Potato virus X-derived vector (pPVX201). (b) The obtained construct was used to infect *N. benthamiana* plants. Infection spread systemically from inoculated leaves to apical leaves, where typical PVX-infection symptoms appeared 7–10 days post-inoculation. (c) Immunoblotting performed with a specific anti-N antibody showed the presence of the N protein (50 kDa) in both inoculated (lane 2) and symptomatic apical leaves (lane 4), but not in non-infected leaves (lane 3). The N protein produced in bacteria (lane 1) presents several degradation products (extra bands)

viding an adequate instrument to develop a rapid, low-cost, immune-based diagnostic assay to be used as an alternative or in association to molecular diagnosis.

For the M protein, we demonstrated that the wild type protein is toxic when expressed in bacteria and only a mutated form was obtained in this system, accumulating in the inclusion bodies. On the contrary, we demonstrated that plants allowed the expression of the full-length original M protein. In particular, we obtained a soluble M protein in *N. benthamiana* plants, using *Agrobacterium tumefaciens*-mediated infection. The reduced electrophoretic mobility observed for the plant-derived M protein, compared to that produced in bacteria, suggests the presence of glycosylation (the native M protein is N-glycosylated at the fourth residue), provided by this eukaryotic system.

These results provide a proof of principle for using plants as a robust, rapid and flexible production system for protein reagents suitable to face potential recurring SARS-CoV outbreaks.

10.2.2 Improved Genetic Vaccines Including Plant Immune-Modulating Sequences

DNA vaccination represents a new milestone in the technological efforts against infectious diseases, offering many advantages over other vaccine approaches due to simplicity, ease of manufacturing and safety.

DNA vaccines are currently used in veterinary medicine but one of the main problems to be solved for human DNA vaccines (both preventive and therapeutic) is their poor ability to induce an adequate immune response (production of antibodies and/or cell-mediated responses).

Several strategies have been developed to improve DNA vaccine efficacy (i.e. codon optimization, transfection reagents, routes of administration, adjuvants, combination with heterologous boosts). Increased understanding of molecular events driving innate and adaptive immune responses has assisted development of molecular adjuvants for DNA vaccine use. Such adjuvants comprise plasmid-encoded signalling molecules including cytokines, chemokines, immune costimulatory molecules, toll-like receptor agonists or inhibitors of immune suppressive pathways [42].

Another possibility of DNA immunization in combatting the threat of emerging infectious diseases, is to offer a unique and powerful approach to the production of high-quality antibodies (polyclonal, monoclonal or recombinant antibodies from phage display libraries) against various pathogens [43]. Compared with traditional protein-based immunization approaches, DNA immunization is efficient for testing novel immunogen design, does not require the production or purification of proteins from a pathogen or the use of recombinant protein technology and is effective at generating antibodies against conformation-sensitive targets.

Recent clinical data have shown that novel DNA vaccines design are able to induce high-level antigen-specific antibody responses [44] but the search of innovative immune-stimulatory sequences with few clinical use constraints (i.e., possible auto-immune responses induced by proteins of human origin) is still an open field.

In the following, examples of successful use of sequences of plant origin as immune-enhancers with the purpose of reinventing vaccine design are reported.

10.2.2.1 Case Study 2: Plant Derived Sequences for Improved Genetic Vaccines Against Infectious Agents

Several years ago we demonstrated that therapeutic (anti-cancer) DNA vaccines can be potentiated by using plant immune-modulating sequences. The driving idea was that some plant proteins, involved in plant defence responses (due to some similarities between mammalian and plant immune mechanisms) might have effects on tumours and human immunity through modulation of innate immune functions. This turned out to be possibly true and induced a tumour-Specific Antigen (TSA)-linked adaptive cell-mediated immunity (crucial for cancer resolution) [45, 46].

Recently, we developed a genetic vaccine where a plant protein signal sequence (ss-), was fused to the N-terminal portion of crucial viral antigens derived from the human papillomavirus type 16, HPV 16 (synthetic/fusion genes derived from E7 and L2 proteins) [47, 48].

Mammalian cells (HEK-293) were transfected to study the transient expression and the intracellular fate of the proteins encoded by the novel DNA constructs. In the case of a ss-E7 construct, the protein was found in the culture medium of transfected cells, whereas E7 without ss- was only present in the cell lysates, demonstrating the ability of the plant signal sequence to modulate the sorting of a heterologous protein in mammalian cells. The plant ss- was found to modify the processing also of other constructs (i.e. ss-E7-CP, where the E7 gene is fused to the coat protein of potato virus X), even though secretion was not observed in the culture medium, while for ss-L2 the protein was detected mostly into the cytoplasm of transfected cells.

The immunological effects of the ss- provided DNA vaccines were studied in animal models for HPV (C57BL/6 mice) with a prime/boost schedule, implying the use of electroporation (EP) after intra-muscular immunization, demonstrating that the plant signal sequence enhances the humoral response to DNA-based vaccines.

Electroporation (EP), indeed, appears a promising approach for improving immunogenicity of DNA vaccines for its ability to increase cellular permeability resulting in a high level of protein expression and improved immune response, as recent clinical trials have shown [49, 50]. A vaccination schedule, comprising a 'prime' with DNA plasmid at time zero and 'boost' with DNA at 1-week interval by intradermal (ID) + EP immunization, resulted in the induction of a strong humoral immune response, confirming that ID + EP is more efficient than intra-muscular (IM) vaccination. In particular, the ss-L2₁₋₂₀₀-E7 plasmid was able to produce the

highest titers of both anti-L2₁₋₂₀₀ and anti-E7 IgGs. The EP immunization protocol determined also a longstanding humoral immune response against L₂₁₋₂₀₀, persisting, at least, 6 months in the utilized mouse model. Preliminary experiments seem to indicate the neutralizing nature of the anti-L2 antibodies.

To our best knowledge, this is the first demonstration that a signal sequence of a plant protein may exert a biological activity in mammalian cells and enhance immunogenicity of an antigen of interest. This approach might work also for other antigens (even if relatively large or glycoproteins) and for different pathogens, opening new perspectives in the design of DNA vaccines, especially to counteract infections where a fast and effective humoral response is needed. Such genetic vaccines can be easily produced on an industrial scale according to GMP, providing more effective and safe vaccines that do not involve the production of chemo/cytokines which might induce secondary responses, or of animal antigens that could cause cross autoimmune responses.

10.3 Conclusions and Perspectives

In order to avoid the devastating loss of life by (possible) viral outbreaks such as a next Ebola, Zika, avian flu, MERS [51] or a biological warfare (BW) attack, whose epidemiology is associated to sudden and unforeseen contagious burst, it is necessary to rely on small stockpiles ready when the next outbreak begins. At the same time it is fundamental to invest in technical platforms able to cut down the time to tailor the eventual vaccine candidate to be effective to the epidemic. In other words, when outbreaks happen, the vaccines will be ready in just few weeks/months for field-testing and mass-manufacture.

The two platforms we introduced, plants as bioreactors for the production of biopharmaceuticals ('Plant Molecular Farming'), and improved genetic vaccines endowed of plant sequences with immune-modulating activity, represent two promising (and complementary) approaches for the rapid and affordable production of countermeasures (diagnostics and vaccine candidates) against emerging, re-emerging and bioterrorism-related infections.

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