Vaccine 27 (2009) 3463–3466

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Plants as biofactories for the production of subunit vaccines against bio-security-related bacteria and viruses☆

M. Manuela Rigano[a], Carmela Manna[b], Anna Giulini[c], Alessandro Vitale[c], Teodoro Cardi[b,*]

[a] Department of Soil, Plant, Environmental and Animal Production Sciences, University of Naples, Via Università 100, 80055 Portici, Italy
[b] CNR-IGV, Institute of Plant Genetics, Res. Div. Portici, Via Università 133, 80055 Portici, Italy
[c] CNR-IBBA, Istituto di Biologia e Biotecnologia Agraria, Via Bassini 15, 20133 Milano, Italy

ARTICLE INFO

Article history:
Available online 2 February 2009

Keywords:
Transgenic plant
Bioterrorism
Vaccine

ABSTRACT

The development of new generation vaccines is an imperative tool to counteract accidental or intended release of bio-threat agents, such as *Bacillus anthracis*, *Yersinia pestis* and variola virus, and to control natural outbreaks. In the past few years, numerous data accumulated on the immunogenicity and safety of plant-made vaccines against bio-security-related organisms. In addition, expression levels achieved for these antigenic proteins are practical for the production of sufficient material for large-scale vaccination programs. These data demonstrated that the plant-based approach is feasible for manufacturing recombinant vaccines against bio-terror agents that could be mass-produced at reasonable cost.

1. Introduction

Bioterrorism is defined as the deliberate use of microorganisms or toxins derived from living organisms to cause death or disease in humans and animals [1]. The ~200 pathogens reported to be potential agents for bioterrorism are classified into three different categories according to the infectiousness, virulence, severity of impact on human health, and cost [1]. The Centers for Disease Control (CDC) lists as "Category A biological agents" pathogens such as *Bacillus anthracis*, *Yersinia pestis* and variola virus. The most effective way to prevent the use of biological agents as a weapon would be to produce efficacious vaccines; however, traditional vaccines are inappropriate for rapid widespread administration following a terrorist attack. Transgenic plants that express recombinant proteins are economical and convenient vehicles to produce protective antigens (PAs). Plant-derived subunit vaccines lack contamination with animal pathogens, are heat stable and may be engineered to contain multiple antigens. In addition, transgenic plants have low raw material cost and enable rapid scale-up capacity. Selected plant tissues are also suitable for oral administration, thus reducing pain and discomfort, eliminating needle-associated risks, and inducing mucosal immunity. We present an overview of expression of target antigens produced in plants for the development of vaccines against bio-threat agents, such as *B. anthracis*, *Y. pestis* and variola virus.

2. Approaches to produce vaccines in plants

Several approaches have been used to produce subunit vaccines, including those against bio-threat agents, in plants (Table 1). To date, the majority of plant-derived pharmaceuticals have been produced by nuclear transformation, wherein the gene of interest is incorporated into the plant nuclear genome. However, random integration of foreign gene via non-homologous recombination results in transformed lines with high variability of transgene expression. In addition, expressed sequences can be subjected to gene silencing, and the foreign proteins are often rapidly degraded in plant tissues [2]. The average protein accumulation levels achieved (0.01–0.4% TSP) are too low for cost-effective production of molecules that can generate a protective immune response, and induce tolerance in individuals [3]. Nevertheless, nuclear transformation remains the most versatile technology, as many species are amenable to transformation and it is possible to specify in which tissue the protein should accumulate [4]. Moreover, the recombinant protein can be targeted to a specific subcellular organelle or compartment, thus increasing expression and allowing post-translational modifications, such as glycosylation [5,6].

A promising alternative for plant-based expression of biopharmaceuticals is to integrate target genes into the plastid genome. Once stably integrated, transgenes express large amounts of proteins due to the high copy number of the chloroplast genome in each plant cell [7]. Chloroplast transformation offers several advantage over nuclear transformation, including uniform transgene expression rates, no gene silencing and gene/product containment. On the other hand, only few species are amenable to plastid transformation and plastid expression offers fewer alternatives

☆ Contribution no. 329 CNR-IGV Portici.

* Corresponding author. Tel.: +39 081 2539223; fax: +39 081 2539481.
E-mail address: cardi@unina.it (T. Cardi).

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doi:10.1016/j.vaccine.2009.01.120

Table 1
Examples of bio-security-related antigen production by different plant-based technologies.

Target	Antigen	Technology	Expression level	Reference
Bacillus anthracis	PA	Chloroplast transformation (tobacco)	14.2% TSP[a]	[14]
B. anthracis	PAD4, LFD1	Transient expression (*Nicotiana benthamiana*)	N.D.	[15]
B. anthracis	CTB-LF	Nuclear transformation (potato)	0.002% TSP[a]	[12]
B. anthracis	PA	Nuclear transformation (tomato)	N.D.	[3]
Yersinia pestis	F1, V, F1–V	Transient expression (*N. benthamiana*)	2 mg/g FW[b]	[17]
Y. pestis	F1–V	Transient expression (*N. benthamiana*)	N.D.	[18]
Y. pestis	F1–V	Nuclear transformation (tomato)	4% TSP[a]	[16]
Y. pestis	F1–V	Chloroplast transformation (tobacco)	14.8% TSP[a]	[7]
Variola virus	B5[c]	Transient expression (*N. benthamiana*)	100 mg/kg (freeze-dried material)	[22]
Variola virus	A27L[c]	Nuclear and chloroplast transformation (tobacco)	0.04% (nuclear) and 18% (chloroplast) TSP[a]	Rigano et al. (unpublished) [20]

[a] TSP, total soluble protein.
[b] FW, fresh weight.
[c] From vaccinia virus.

for post-translational modifications. However, recent studies have shown that protein oligomerization, disulphide-bond formation and lipidation can occur also in the chloroplast [2,8,9].

An alternative approach for producing large quantities of vaccine antigens is to transiently express proteins from recombinant plant viral sequences inoculated onto plants. This technique provides an ideal strategy to produce recombinant molecules in a short period of time. Typically, recombinant proteins have been expressed using plant viral RNA vectors. Recently, hybrid vectors that combine elements of plant RNA viral vectors and *Agrobacterium* binary plasmids were developed to enable uniformly high levels of target protein expression and rapid scaleup [10].

3. Plant-based vaccines against bio-security-related organisms

3.1. Anthrax

B. anthracis is the causative agent of anthrax. Anthrax is considered to be one of the most potent bio-terror agents, as *B. anthracis* spores can be transmitted by aerosolization. Anthrax inhalation has a high mortality rate, and the organism's spores are quite stable in the environment [1]. Vaccination against anthrax has been considered the most effective prophylactic measure. However, the current licensed anthrax vaccine is a cell-free preparation derived from an attenuated *B. anthracis* strain in which production process is complicated and imprecise. Moreover, the vaccine requires an extended immunization program and has a relatively high incidence of side effects [4]. New generation subunit vaccines against anthrax have targeted the components of the multicomponent lethal toxin, which consist of the PA and lethal factor (LF). The first reports of PA expression in plants were based on nuclear-transgenic tobacco, potato and tomato plants. The tobacco-made PA was biologically active as was demonstrated through its ability to lyse a macrophage cell line *in vitro* when combined with LF [11]. Also, mice immunized with the tomato-expressed PA generated lethal toxin neutralizing antibodies [3]. For the production of an edible anthrax vaccine, Kim et al. [12] generated potato plants expressing detectable amount of cholera toxin B-subunit – anthrax LF conjugate fusion protein [12]. In an effort to produce an anthrax vaccine in large quantities, PA was expressed in transgenic tobacco chloroplasts [13,14]. It has been calculated that one acre of chloroplast transgenic plants could produce up to 360 million doses of anthrax vaccine antigens [14]. Subcutaneous immunization of mice with partially purified chloroplast-derived or *B. anthracis*-derived PA with adjuvant yielded immunoglobulin G titers up to 1:320,000, and both groups of mice survived challenge with lethal doses of toxin. In a separate study, Chichester et al. [15] described a candidate subunit vaccine against *B. anthracis* consisting of domain 4 of PA (PAD4) and domain 1 of LF (LFD1). Each domain was fused to lichenase, a thermostable enzyme from *Clostridium thermocellum*, and transiently expressed in *Nicotiana benthamiana* using the "launch vector" technology that combines elements of plant virus RNA vectors and *Agrobacterium* binary plasmid [10]. Immunization of mice with this candidate vaccine resulted in high titers of lethal toxin neutralizing antibodies.

3.2. Plague

Y. pestis is the causative agent of plague that can occur in bubonic or pneumonic forms. Bacteria are usually passed to humans through the bite of a flea or by direct contact with, or inhalation of, infective materials. *Y. pestis* appears to be a good candidate agent for a bioterrorist attack, because of the rapid spread of the disease from person to person as a consequence of the use of an aerosolized form of this agent [1,16]. Currently, there is no safe and effective vaccine against plague. Two *Y. pestis* antigens are of interest for developing new vaccines against plague: the anti-phagocytic capsular envelope glycoprotein (F1) and the low calcium response virulent antigen (V). In one study, the antigens F1, V, and the fusion protein F1–V were produced by transient expression in *N. benthamiana* using a deconstructed tobacco mosaic virus-based system. All the plant-derived antigens, administered s.c. to guinea pigs, elicited antigen-specific serum IgG titers and provided protection against an aerosol challenge of virulent *Y. pestis* [17]. Using a similar transient system, Mett et al. [18] expressed the antigens F1 and V as fusions with lichenase. When administered to Cynomolgus Macaques, the purified plant-produced antigens stimulated strong immune responses and provided complete protection against lethal challenge with *Y. pestis* [18]. In a separate study, the F1–V antigen fusion protein was expressed in transgenic tomato plants for the development of an alternative oral subunit vaccine against plague. The immunogenicity of the plant-made vaccine was tested in mice that were primed subcutaneously using bacterially produced F1–V and boosted orally with freeze-dried F1–V transgenic tomato fruits. The vaccine elicited IgG1 in serum and mucosal IgA in fecal pellets [16]. In a different study, the fusion protein F1–V was expressed in transgenic tobacco chloroplasts. The maximum expression levels of the fusion protein were observed in mature leaves and was as high as 14.8% TSP [7].

3.3. Smallpox

Variola virus, which belongs to the family of *Poxviridae*, genus othopoxvirus, is the causative agent of smallpox. The aerosol infectivity, high mortality and stability of the variola virus make it a potential and dangerous threat in biological warfare. In addition, animal poxviruses such as monkeypox, and recombinant variants of the variola virus, could be developed as biological weapons [1]. The existing smallpox vaccine, i.e. scarification with vaccinia virus

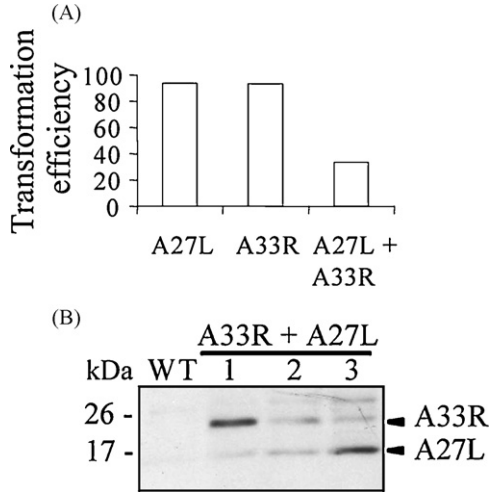

Fig. 1. Stable nuclear expression of VACV proteins in tobacco plants. A sequence encoding the FLAG epitope was added at the C-terminus of the *A27L* and *A33R* coding sequences. The constructs were cloned in binary vectors for *Agrobacterium*-mediated transformation. (A) Efficiency of transformation (no. of transgenic shoots/100 co-cultivated explants) with the binary vectors containing either *A27L* or *A33R*, and efficiency of co-transformation with the two binary vectors. (B) Co-expression of A33R and A27L recombinant polypeptides in three independent transgenic tobacco plants (1–3). Total protein extracts from leaves were analyzed by SDS-PAGE and protein blot using anti-FLAG antiserum. WT, protein extracts from wild-type plants.

(VACV), is extremely effective but has a high reactogenicity with adverse cardiac events and a wide range of contraindications [19]. Vaccine stockpiles are also low, since smallpox vaccine production ceased in 1980. Good molecular candidates for the development of a smallpox vaccine are the A27L and L1R proteins, specific to the intracellular mature virus (IMV), and the A33R and B5R proteins, specific to the extracellular enveloped virus (EEV). In our laboratories, we are investigating the possibility to produce the above-mentioned four immunogenic proteins of VACV in tobacco. We first performed transient expression of A27L, A33R, L1R and B5R in tobacco protoplasts and verified protein synthesis and stability in pulse-chase experiments. A33R and A27L proteins were synthesized at much higher levels than the others, A27L being also more stable than A33R [20]. Having verified the suitability for plant expression, we stably expressed the A27L and the A33R protein by *Agrobacterium*-mediated transformation of the nuclear genome and biolistic transformation of the plastome. The integration of the *A27L* gene into the chloroplast genome resulted in high-yield accumulation of the recombinant protein (Rigano et al., unpublished, Table 1). In addition, since optimal protection to the challenge is provided by a multicomponent smallpox vaccine [21], *Agrobacterium*-mediated co-transformation experiments with the *A33R* and *A27L* genes are under way. Preliminary results showed high frequency of co-transformation and co-expression of the two proteins (Fig. 1) (Rigano et al., unpublished). *In planta* production of the recombinant vaccinia virus B5 antigenic domain (B5) was reported by Golovkin et al. [22]. The antigen was produced in soluble and insoluble forms upon transient and stable plant transformation. Intranasal administration of soluble B5 led to a rise of B5-specific immunoglobulins. Mice immunized i.m. with B5 generated an antibody response that conferred protection from challenge with a lethal dose of vaccinia virus [22]. The same authors analyzed the impact of purity of plant-based B5 subunit vaccine preparation on specific antibody responses in mice, and demonstrated the efficiency of mucosal administration of plant-derived smallpox vaccine in obtaining a potent immune response [23].

4. Conclusion

The potential use of orthopoxvirus (OPV) in bioterrorism, the occurrence of zoonotic OPV and SARS outbreaks, and the deliberate release of *B. anthracis* in the United States in 2002, highlighted the importance of the development of new generation vaccines against bio-security-related organisms. The use of plants as bioreactors for manufacturing high-volume reserves of subunit vaccines is an economical and safe alternative to the facilities required for yeast, *Escherichia coli*, and mammalian cells [16]. During the last decade, a substantial amount of data has been accumulated on the immunogenity and safety of plant-made vaccines. Moreover, there have been significant advances in improving the yield achieved for many plant-derived antigens. However, several challenges remain to be met in terms of social acceptability of plant-produced vaccines, establishing regulatory guidelines for plant-produced materials and improving the quality of the resulting products. Future studies will also likely focus on the need to fully separate recombinant antigens from potentially harmful secondary metabolites [4]. Downstream purification costs will also need to be reduced, to fully exploit the advantages associated with the use of transgenic plants as bioreactors [24]. However, the growing number of antigens being expressed in plant systems and the six plant-produced vaccine candidates evaluated in Phase I human clinical trials [10] are moving us closer to the production of safe plant-made vaccines against emerging infectious diseases or bioterrorism agents.

Acknowledgements

This research was partially supported by grants from Italian Istituto Superiore di Sanità (ISS)-Istituto Nazionale Malattie Infettive "L. Spallanzani" (INMI) "II anno Programmi di ricerca sulle malattie infettive di grande rilievo sociale e causate da agenti di possibile utilizzo come armi non convenzionali (agenti virali)", and from Italian Ministry of Research (MIUR) "Incentivazione alla mobilità di studiosi stranieri e italiani residenti all'estero".

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