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Developing Country Applications of Molecular Farming: Case Studies in South Africa and Argentina

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Abstract: Molecular farming is a technology that is very well suited to being applied in developing countries, given the reasonably high level of expertise in recombinant plant development in many centers. In addition, there is an urgent need for products such as inexpensive vaccines and therapeutics for livestock and for some human diseases – and especially those that do not occur or are rare in developed regions. South Africa and Argentina have been at the fore in this area among developing nations, as researchers have been able to use plants to produce experimental therapeutics such as nanoantibodies against rotavirus and vaccines against a wide variety of diseases, including Rabbit haemorrhagic disease virus, Foot and mouth disease virus, Bovine viral diarrhoea virus, bovine rotaviruses, Newcastle disease virus, rabbit and human papillomaviruses, Bluetongue virus, and Beak and feather disease virus of psittacines. A combination of fortuitous scientific expertise in both places, coupled with association with veterinary and human disease research centers, has enabled the growth of research groups that have managed to compete successfully with others in Europe and the USA and elsewhere, to advance this field. This review will cover relevant work from both South Africa and Argentina, as well as a discussion about the perspectives in this field for developing nations.

Keywords: vaccines, therapeutics, biofarming, rotavirus, foot and mouth disease, papillomavirus, nanoantibody, Newcastle disease, beak and feather disease, policy, plant production

INTRODUCTION

In the early days of biopharming, when the proposed use of transgenic plants as cheap edible vaccines was the order of the day, much was made of the prospect of developing countries using the technology to produce vaccines and other biologics on site, where they were to be used. Purported advantages over use of conventional vaccines and vaccination policies included needle-free delivery, lack of necessity for a cold chain, and the very low-cost delivery of vaccines and therapies, especially for "orphan" diseases not common in developed countries [1-4].

Sadly, the original and ambitious goal of cheap, needle-free, edible vaccines seems as far away now as it was in the 1990s, as the unpleasant realities of regulatory approval, quality and dose control became apparent. It now appears to be common cause that even though oral dosing is still a desirable feature, plant-made products will have to be processed for uniformity, formulated in a reproducible way, and given under supervision for best efficacy [5].

The application of the technology has also not yet quite moved into the realm of public acceptability. While a plant cell-made Newcastle disease fowl vaccine was passed by the USA Food and Drug Administration and a plant-made hepatitis B surface antigen (HBsAg) monoclonal antibody by the Cuban authorities as long ago as 2006, there is currently only one therapeutic product very recently registered for public delivery – and that is a lucrative niche product enzyme (glucocerebrosidase, or taliglucerase alfa) intended for the relatively few global victims of Gaucher disease, approved by the FDA in May 2012 [6].

Even sadder has been the very obvious lack of developing country involvement in the area: at the Third Plant-Based Vaccines and Antibodies (PBVA) meeting in Verona in 2009, for example, just 16 out of 180 delegates came from developing countries, with an even lower proportion of presentations [7]. The 2011 meeting was even worse: just 6 of 119 listed delegates were from a developing country, and then from relatively advanced nations such as South Africa, Argentina, Malaysia, Brazil and China (http://www.meetingsmanagement.com/pbva_2011/).

Thus, while the uses and applications of biofarming technologies have always been touted as being ideal for developing countries, neither the technologies nor even the products of developed nation research have actually reached those parts of the world in the shape of vaccines or therapeutics to any meaningful extent. There are no plant-made animal vaccines in common use in Africa, South America or Asia; no cancer therapeutics have been registered or even tested there; there is hardly any funding available for biofarming projects even in the few developing countries that have helped pioneer the science, like Argentina and South Africa (this review, and [8]).

So just what are the developing country applications of the title, given what appears from the above to be a gloomy current global outlook for biofarming? Perhaps fortunately, for what represents a considerable proportion of the human race, as well as its agricultural and domestic livestock, we think that the applications and prospects for biofarming are in fact very promising. Internationally, products are being tested in clinical trials, and regulatory aspects are definitely improving [9-11]; new technologies and better plant expression vectors are continually being tested [12-14] – and major initiatives are under way that have great promise for the whole field. While they undoubtedly lag in application and legislation, developing countries too have good prospects.

For example, South Africa and Argentina are countries with a high capacity for production of foods of plant and animal origin. Veterinary vaccines are undoubtedly an essential tool to prevent animal diseases, and are therefore a critical issue to optimize animal production. This fact, and the lower complexity of regulatory processes for the development of veterinary vaccines in comparison with the human pharma, are the reason why our groups have al-

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ready or are now targetting the use of molecular farming in this area.

Another niche to exploit is the production of industrial enzymes. These are normally required in large quantities at low cost and with a relatively low degree of purification – so in this case, the use of plants as bioreactors becomes a very promising strategy. A further niche would be the replacement of high-cost bovine carcass-derived reagents, such as pancreatic enzymes: the ever-present fear of bovine spongiform encephalopathy (BSE) transmission increasingly makes this a very attractive proposition [8].

In 2007, Argentina set up the Ministry of Science, Technology and Productive Innovation (MINCyT). This country and Brazil are unique in Latin America in having obtained governmental support to develop scientific and technological projects with the purpose of strengthening the productive model so as to generate a better social inclusion and to improve their own economies. In South Africa, the recent creation of the Technology Innovation Agency has given hope for a renewed emphasis on biotechnology funding – and a recent national Department of Science and Technology push to get funding plans and strategies in place for both molecular farming and vaccine production in general, is highly promising.

Thus, the success of biofarming in developing countries, in the absence of major private funding initiatives or a well established commercial molecular farming infrastructure, will probably be associated with governmental decisions to support the establishment of biofarming platforms, and the development of appropriate scientific expertise and the detection of niches to be exploited.

This review, therefore, will discuss biofarming applications for developing countries with a particular bias on developments in Argentina and South Africa, but will attempt to generalize these examples to the rest of the developing world.

EXAMPLES OF DEVELOPMENTS IN ARGENTINA

INTA: National Institute of Agricultural Technology

The National Institute of Agricultural Technology (INTA) in Argentina has a strong background in developing plant-based immunogens as an alternative strategy to conventional vaccines. The main antigens that have been used in molecular farming are from viral pathogens which affect both poultry and cattle.

The first activities in molecular farming started in the Institute of Virology (IV) and the Institute of Genetics (IG) in the mid-1990s with the aim of evaluating transgenic plants as a source of antigen for veterinary vaccines. The expertise base in the IG includes plant transformation via recombinant *Agrobacterium tumefaciens*, regeneration and cultivation of transgenic alfalfa and potato plants, biolistic transformation and regeneration of maize, alfalfa, potatoes, wheat and soybeans. The IV has since 1980 investigated different topics related to viral diseases that affect farm animals, and has become a reference center for pathogens such as Foot and mouth disease virus (FMDV), Bovine rotavirus (BRV), Bovine viral diarrhoea virus (BVDV), Bovine leukemia virus (BLV) and several other equine and avian viruses.

Within that scope, the structural protein VP1 from FMDV, which carries critical epitopes responsible for the induction of protective neutralizing antibodies, was used as a model. It was demonstrated that VP1 could be successfully expressed in *Arabidopsis thaliana*, alfalfa and potato plants [15-18], and that those materials elicited an antibody response and protection against the virulent challenge when parenterally or orally administered in mice as experimental immunogens.

A subsequent approach was to express the capsid P1-2A and the protease 3C coding regions, necessary for processing P1 to the four capsid proteins, in alfalfa plants: these have a high protein content and low level of secondary metabolites, make them suitable for generating recombinant proteins. In addition, alfalfa can be propagated by stem cuttings, allowing rapid scaling up of the product. It is well known that FMDV empty capsids maintain continuous and discontinuous B-cell epitopes presented in the authentic virion as well as T-cell epitopes identified in cattle and swine [18], and are capable of eliciting the same qualitative antibody response as infectious FMDV particles [19]. The expressed products induced a strong FMDV specific antibody response against complete virus particles and viral subunits as well as a complete protection against the experimental challenge with the virulent virus [20].

Nevertheless, the expression level of the recombinant proteins in transgenic plant tissues was relatively poor, a limitation also found by several other groups working in molecular farming.

A potential solution to this problem was selecting those transgenic events expressing exceptionally high levels of the recombinant protein. For this purpose, the group developed a methodology based on the construction of a fusion protein composed of a very well known and easily detectable reporter gene, β-glucuronidase (*gusA*), fused to an epitope of interest. This strategy allowed the succesful expression of an epitope from FMDV VP1 protein and an epitope from BRV VP4 protein [21,22].

An alternative strategy explored by the group in collaboration with J. Morris from the University of Nebraska, was the use of a plant viral vector: this was used to explore transient expression in *N. benthamiana* of the BRV VP8 protein. High expression levels were achieved, and the recombinant protein purified from tobacco leaves elicited a protective passive immune response, assessed in the suckling mouse model where antibodies from the dam protected against infection [23]. Glycoprotein D from Bovine herpesvirus 1 and FMDV VP1 were also efficiently expressed using this methodology, and evoked protective immune responses [17,24].

Another alternative to overcome the low expression level was to increase the vaccine immunogenicity by increasing the number of MHC-peptide complexes on the surface of antigen presenting cells (APCs). This could be done by fusing antigens to specific antibodies against APCs´ surface markers. To test this hypothesis, a truncated version of BVDV glycoprotein E2 without the transmembrane domain (tE2) was fused to a single-chain antibody against MHCII (APCH-tE2). When evaluated in guinea pigs, the fusion protein (APCH-tE2) was able to elicit the same level of neutralizing antibodies as the single protein did (tE2), but with at least five times less antigen. In cattle, the subunit vaccine elicited BVDV-specific neutralizing antibodies and afforded complete protection after challenge [25].

INGEBI – CONICET: Engineering Research Institute in Genetics and Molecular Biology

The Plant Virology and Biotechnology group led by F. Bravo has been investigating chloroplast transformation since 2006 [26]. Transplastomic plants have an extraordinary potential for antigen production in plants due to their ability to accumulate high levels of recombinant proteins. Moreover, there is increased biosafety since plastid inheritance in most crops is only via the maternal line. Their first work with this platform was the production of a fusion protein between the β-glucuronidase reporter gene (*gusA*) and the highly immunogenic epitope (site A) of the structural protein VP1 of the FMDV. The FMDV epitope expressed in transplastomic plants was immunogenic in mice [27]. The group has also produced the C486 BRV VP8* protein in tobacco chloroplasts. VP8* plant extracts elicited a strong immune response in female mice which was passively transferred to the offspring [12]. Recently, different strategies were evaluated to improve the accumulation of a neutralizing VHH antibody against rotavirus in transplastomic tobacco plants. The conclusion of that work was that VHH could be successfully obtained either in the thylakoid lumen or as a fusion protein with β-glucuronidase [28].

IIB-INTECH: Biological Research Institute - Technological Institute of Chascomús

This laboratory has been working on different strategies of expression of recombinant antigens so as to use plants as bioreactors. They evaluated the feasibility of using either transgenic plant or *Agrobacterium*-mediated transient expression for the production of recombinant antigens as oral vaccines against *Toxoplasma gondii* (Cóceres *et al*., 2010; Laguia Becher *et al*., 2010), and *Leishmania* spp. They are also studying the value of Hsp90 as a "carrier" of peptides of interest and as a proteinase inhibitor for the optimization of the expression of heterologous proteins in *A. thaliana* and *N. benthamiana* (AtHsp90 and NbHsp90) (Corigliano *et al*., 2011).

FCEN–UBA: University of Buenos Aires.

The group of A. Mentaberry has much experience in working with recombinant Potato virus X (PVX), and the expression of proteins in *Nicotiana tabacum* using PVX-based vectors. They expressed the *Mycobacterium tuberculosis* complete ESAT-6 open reading frame as a fusion protein with the 2A peptide of FMDV and the amino terminal end of the PVX coat protein (CP) (PVXESAT-6). This strategy allowed the expression of both free CP and ESAT-6 and ESAT-2A-CP fusion protein in the surface of recombinant chimaeric virions to be used as particulate antigen in vaccination (Zelada *et al*., 2006).

Center for Science and Technology Dr. César Milstein - CONICET - Pablo Cassará Fundation.

In 2004 the National Research Laboratory was created with the purpose of contributing to the development of life sciences, biotechnology and health through the development of research programmes intended for the resolution of social or economic problems. The molecular farming group has been involved in investigating "phytofermentation" processes for producing the catalytic antibody 14D9 in *in vitro* cultures of *N. tabacum*. This antibody catalyzes the protonation of prochiral enol ethers with high enantioselectivity (>99% *ee*) and a practical turnover rate (k_{cat} = 0.4 s^{-1}), allowing for preparative scale applications. This antibody represents one of the rare examples of catalytic antibodies promoting acid-catalyzed processes. They have been also working in biotransformation as a tool for obtaining drugs for pharmaceutical use, such as the production of scopolamine. In addition the group has recently evaluated the expression of veterinary antigens in *Nicotiana tabacum* (Nelson et. al., 2012).

INDEAR- CONICET

INDEAR is the research and development company belonging to Bioceres SA: it is the first company created by soy producers to develop, and not just adopt, solutions to problems faced by entrepreneurs who want to participate in the biofarming revolution.

Molecular farming projects are targeted primarily to the production of industrial enzymes in safflower seeds. INDEAR recently signed a production and commercialization agreement with the Canadian biotech company SemBioSys Genetics Inc (SBS): this company developed a cutting-edge technology to produce recombinant proteins in safflower seeds accompanied by an important patent portfolio; however, they have recently ceased operations. The portfolio includes the technology for the production of bovine chymosin, an enzyme used in the dairy industry, and cellulose degrading enzymes necessary for the production of second-generation biofuels.

POTENTIAL PRODUCTS FROM ARGENTINA

The following examples of plants expressing antigens from animal pathogens are highlighted as being illustrative of the wide potential of biofarming for providing products for veterinary medicine (Table **1**)

REGULATORY SYSTEM IN ARGENTINA

Since 1991 Argentina has had a regulatory framework for advances and technological developments in agricultural biotechnology. The implementation of the regulations for genetically modified organisms (GMOs) that are evaluated in experimental trials and which could eventually obtain a marketing authorization, ensures their safe use for the agroecosystem and for human consumption. It also regulates the development of the product from the first experimental releases in order to anticipate any unexpected effect that GMOs could produce.

The criteria used for the evaluation of any GMO contemplate the analysis on a case by case basis: each request is evaluated individually, thoroughly considering the particularities of the species, the introduced genes, the expected effect of these and their interaction with the environment where it is expected to be released. All analyses are performed by applying evidence-based scientific judgment. Supporting documents that applicants (developers of events) submit for the evaluation must have the quality of scientific publications.

The concept of familiarity for the analysis of events or similar species and the history of safe use of the event or the expression of the introduced genes is also considered. All these criteria are used for experimentation, testing and approval for the field cultivation of GMOs.

The government office that provides the framework for these activities is the Ministry of Agriculture, Livestock and Fisheries, which regulates all matters relating to GMO species belonging to agricultural use (use in agriculture, livestock, / aquaculture, fisheries, forestry or potentially could be used in an agricultural context).

The legislation that allows the use of the GMOs is regulated by the Department of Biotechnology and the National Advisory Committee on Biotechnology (CONABIA), which defines the conditions to be applied for each submission. The food safety assessment of GMOs is done by the National Health Service and Food Quality (SENASA) and the Technical Advisory Committee on the Use of GMOs (CTAUOGM).

To date, 24 events have been approved for marketing, production and supply (soybeans (3), corn (18) and cotton (3)), all with agronomic interest (resistance to herbicides, insects or combinations of both). These were developed by multinational companies. However, there are many applications from national public institutions (INTA, Obispo Colombres, FAUBA), a private and public organization (INDEAR) and national companies (Nidera, Don Mario) that are in the approved pipeline.

CONABIA has evaluated a large number of trials related to molecular farming, but none of them have passed the experimental stage yet. One of the reasons could be that these developments were done in public institutions without the participation of private investors; consequently, there is a gap in the value chain that has yet to filled by non-government investors, given that they have not yet been involved in the process.

The regulatory criteria to be applied in this case are basically the same as those described above, but according to the molecule concerned, it may be necessary to comply with the rules of the National Drug, Food and Medical Technology (ANMAT) or SENASA.

OPPORTUNITIES FOR MOLECULAR FARMING IN ARGENTINA

As mentioned above, Argentina has a solid regulatory framework for transgenic plants (CONABIA markets SENASA and Management) within the Secretariat of Agriculture, Livestock, Fisheries and Food of the Nation (SAGPyA).

The success of molecular farming is associated with a government decision to support the creation of a platform that allows

Table 1. Examples of host and expression systems for the production of vaccine-related antigens in Argentina

Antigen	Pathogen	Susceptible host	Target animal (*)	Expression host	Expression system	Expression level	Reference
Truncated glycoprotein gD	Bovine Herpes Virus 1	Cattle	Mice Cattle	*Nicotiana benthamiana*	Transient expression / Tobacco mosaic virus	20 ug/g FW[a]	[24]
VP8 protein	Bovine Rotavirus	Cattle	Mice	*Nicotiana benthamiana*	Transient expression / Tobacco mosaic virus	5 ug/g FW	[23]
ESAT-6 protein	*Micobacterium tuberculosis*	Human	-	*Nicotiana tabacum*	PVX virus / transient expression	0.5-1 % TSP	[29]
VP1 protein	Foot and mouth disease virus	Cattle	Mice	alfalfa	Stable expression / *Agrobacterium tumefaciens*	-	[30]
P135-160 peptide from VP1 protein fused to B-glucuronidase	Foot and mouth disease virus	Cattle	Mice	alfalfa	Stable expression / *Agrobacterium tumefaciens*	0.5-1 mg/g TSP	[21]
eBRV4a peptide from Bovine Rotavirus VP8 protein fused to B-glucuronidase	Bovine Rotavirus	Cattle	Mice	alfalfa	Stable expression / *Agrobacterium tumefaciens*	0.4-0.9 mg/g TSP[b]	[22]
Polyprotein P1 and protease 3C	Foot and mouth disease virus	Cattle	Mice	alfalfa	Stable expression / *Agrobacterium tumefaciens*	0.005-0.01% TSP	[20]
Truncated glycoprotein E2 fused to a MHCII targeting molecule	Bovine Viral Diarrhea virus	Cattle	Guinea pig Cattle	alfalfa	Stable expression / *Agrobacterium tumefaciens*	1 ug/g FW	[25]
VP1 protein	Foot and mouth disease virus	Cattle	Mice	*Solanum tuberosum* cv. Desirée	Stable expression / *Agrobacterium tumefaciens*	-	[16]
Fusion protein and hemagglutinin protein	Newcastle Disease Virus	Avian	Mice	Potato leaves	Stable expression / *Agrobacterium tumefaciens*	0.3-0.6 ug/mg total leaf protein	[31]
P135-160 peptide from VP1 protein fused to B-glucuronidase	Foot and mouth disease virus	Cattle	Mice	*Nicotiana benthamiana*	Transplastomic plants	51 % TSP	[27]
VP8 protein	Bovine Rotavirus	Cattle	Mice	*Nicotiana benthamiana*	Transplastomic plants	600 ug/g of fresh tissue (FT)	[12]
VHH fused to B-glucuronidase		-	-	*Nicotiana benthamiana*	Transplastomic plants	3% TSP	[28]
Sruface antigen 1	*Toxoplasma gondii*	Some mammals including human	Mice	*Nicotiana tabacum*	*Agrobacterium*-mediated transient expression	0.06-1 % TSP	[32]
His-tagged truncated version of Toxoplasma gondii dense granule 4 protein (Gra4(163-345))	*Toxoplasma gondii*	Some mammals including human	-	*Nicotiana tabacum*	*Agrobacterium*-mediated Transient transient expression	0.01 % TSP / 0.1 % AWF	[33]

(Table 1) Contd….

Antigen	Pathogen	Susceptible host	Target animal (*)	Expression host	Expression system	Expression level	Reference
HN glycoprotein	Newcastle Disease Virus	Avian	-	*Nicotiana benthamiana*	Agroinfiltration transient expression	3 ug/mg total leaf protein	[34]
Truncated glycoprotein E2	Bovine Viral Diarrhea virus	Cattle		*Nicotiana tabacum*	*Agrobacterium*-mediated Transient transient expression	1.3% TSP	[35]

(*) Experimental animal where the efficacy of the recombinant vaccine / antibody was assessed.
a: fresh weight
b: total soluble protein
c: apoplastic washing fluids

generation of the necessary infrastructure to enhance the scientific knowledge acquired in the last 15 years.

Argentina is in third place in the world in adoption of the use of GMOs by farmers, in terms of the area of transgenic crops under cultivation. It is also relevant that there is strong acceptance of the public of products derived from transgenic plants. However, to date there is no network covering molecular farming platforms in Argentina.

SOUTH AFRICA AND AFRICA

The development of molecular biotechnology in South Africa followed closely on developments in the developed world: by 1999, for example, a review by one of us reported that a number of parastatal organisations, companies and universities had significant capacity for and involvement in plant transformation and other related activities, and that "*…the sole factor currently limiting biotechnology in South Africa is funding: very few private companies do or fund molecular biotechnology research (apart from research at the dedicated Institutes), and government funding is very limited*" [36]. While this has not yet changed significantly, significant progress in molecular biotechnology and especially biofarming was made in the interim, with funding from a variety of sources. Biofarming research was initially limited to the Rybicki laboratory, which has been involved in this field from the mid-1990s; however, this has recently expanded to include a number of others, including in the neighbouring country of Botswana. The involvement of other South African institutions has been reviewed in detail in 2012 [8]; this review will therefore target only relevant recent developments not covered there.

University of Cape Town

The Subunit Vaccines Group in the Institute of Infectious Disease and Molecular Medicine (IIDMM) and Department of Molecular & Cell Biology at the University of Cape Town has been involved in investigating plant production of Human papillomavirus (HPV) vaccines since 1995, and was involved in Human immunodeficiency virus subtype 1 (HIV-1) vaccine research from 2000-2009. They have also been involved in projects involving vaccines to Beak and feather disease circovirus (BFDV) of parrots, H5N1 highly pathogenic influenza virus, a South African isolate of human rotavirus and Bluetongue orbivirus, as well as in the contract production of single-chain variable region antibodies (scFvs) derived from a chicken IgY phage display library. The lab can presently handle benchtop through to greenhouse-scale production, processing and purification, and has an expertise base which includes plant transformation via recombinant *Agrobacterium tumefaciens* and biolistic transformation, regeneration and cultivation of transgenic *Nicotiana* spp. as well as of *Zea mays*, transient expression via small- and large-scale (syringe or vacuum) infiltration of *Nicotiana* plants with *A tumefaciens*. They also have insect cell culture facilities and regularly uses recombinant baculoviruses for protein expression, as well as using a variety of protein concentration and purification platforms and immunoassays for proving expression (see [37] for a recent detailed review).

The group has a systematic approach to expressing new antigens, which involves the use of simple *Agrobacterium tumefaciens*-based transient expression in *N benthamiana,* as well as use of replicating and non-replicating plant virus-derived vectors [13,38]. In addition, they systematically investigate a number of expression parameters for every antigen, including different codon use, and intracellular localisation in the cytoplasm, in plastids, retention via KDEL motif in the endoplasmic reticulum, and secretion via the ER to the apoplast. Other means of improving expression including truncation or modification of proteins, and/or fusion to various partners, are also used [37].

Salient examples for the future potential of the laboratory from recent work include the optimization of expression of HPV-16 L1 major capsid protein and derivative vaccines, the establishment of the laboratory as an African center for future plant-based influenza vaccine production, and high-level production of a South African isolate of human rotavirus.

Since the first report of production via transgenic plants in 2003 [39], till the routine production of antigen via transient expression in recent times, the laboratory has managed to increase the yield of HPV-16 L1 major capsid protein by a factor of some 250 000-fold [40] (see Fig. **1**). Use of later chimaeric versions of it, with inclusion of peptides from the minor L2 protein, have increased yields higher still (EP Rybicki, II Hitzeroth, M Whitehead, C Pineo, M Burger, unpublished results). This illustrates the feasibility of significantly improving even initially very poor expression of vaccine antigens, to exploitable levels, by systematic investigation of the necessary parameters. While the two commercially-available HPV vaccines are currently enjoying blockbuster status [41], they are still expensive – and it could be that first or even second-generation HPV vaccines made in plants can provide a genuine alternative for universal vaccination.

The Rybicki group recently reported the successful expression and immunogenicity testing of a haemagglutinin (HA) gene from the highly pathogenic avian influenza H5N1 virus (A/Viet Nam/1194/2004) [42], as part of an intended platform for the rapid-response plant production of pandemic and possibly seasonal influenza vaccines in South Africa. A full-length gene was synthesized with human codon usage, and two versions of it – the original, and one with the transmembrane domain coding region removed – were tested for expression in *N benthamiana* via the same transient agroinfiltration regime used for HPV-16 L1. Following successful expression, transgenic *N tabacum* cv Petit Havana was also regenerated: in both systems, the truncated (H5Tr) and full length (H5) proteins accumulated best in the ER and in the apoplastic space respectively, and yields in excess of 100 mg/kg could be obtained. The H5 protein in particular had a good haemagglutination titre,

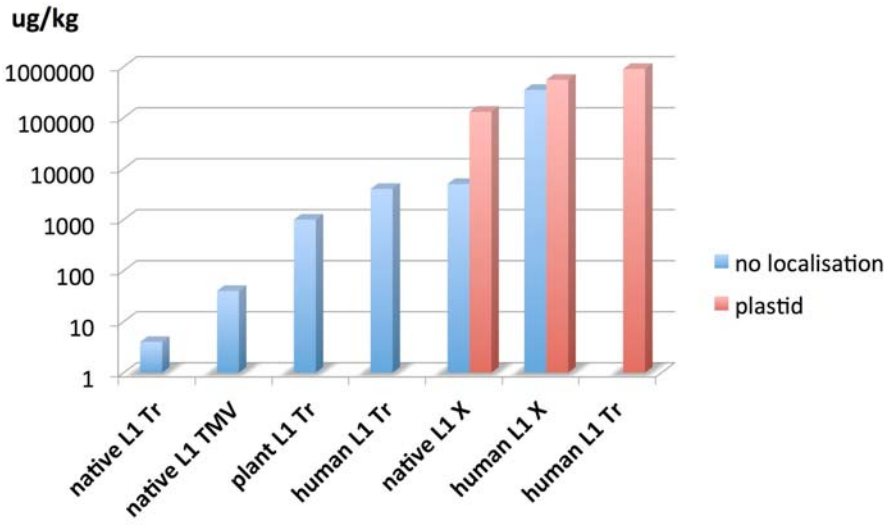

Fig. (1). Yield increases using different optimization strategies. Native L1 Tr = native HPV-16 L1 gene, transgenic *N tabacum* cv Xanthi. Native L1 TMV = expression of native gene in *N benthamiana* via TMV vector. Plant L1 Tr = *Nicotiana*-optimised gene in transgenic *N tabacum* cv Petit Havana. Human L1 Tr = human codon-optimised gene, same host. Native L1 X = transient expression in *N benthamiana* using agroinfiltration. Human L1 X = transient expression, human codon-optimised gene. Human L1 Tr = humanized gene in transgenic *N tabacum* cv Petit Havana. No localisation / plastid = intracellular targeting of protein via signal sequences.

was immunogenic in mice and in chickens, and antisera to it had haemagglutination-inhibition (HI) titres appropriate for virus neutralization – which is a good indication of functionality of the antigen. This success, taken with the prior demonstrations by companies such as Medicago Inc. of the great potential of transient plant expression for rapid-response influenza vaccines [43], means that local implementation of the technology in developing countries is highly feasible.

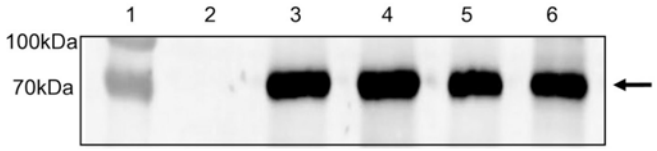

Fig. (2). Western blot analysis of influenza virus haemagglutinin (HA) products harvested from leaf apoplastic space extracts. *N. benthamiana* plants transiently expressing an apoplast-targetting vector were infiltrated with buffer, following which the buffer was collected by low-speed centrifugation from cut leaves. HA was detected in the apoplastic space extracts using rabbit anti-H5N1 antibody. Lane 1, protein ladder; lane 2, wild type plant control; lanes 3 – 6, H5 products of different infiltrated leaf batches. From Mortimer *et al. BMC Biotechnology* 2012 **12**: 14 doi: 10.1186/1472-6750-12-14

The third example from Cape Town is the expression of the capsid proteins of a local and southern Africa-prevalent isolate of human rotavirus (G9 P[6]) that is not well matched to available commercial vaccines. The Department of Science and Technology of the South African government funded a "novel vaccines" consortium project in South Africa from 2008-2011, that encompassed insect cell / recombinant baculovirus and plant-based production of rotavirus, and plant- and yeast-based production of chimaeric HPV-L1 vaccines. Rotavirus capsid protein (VP2, VP4, VP6 and VP7) expression in *N. benthamiana* was targeted to the cytosol, endoplasmic reticulum, apoplast and chloroplast. Western blot results showed the successful expression of VP6 in all four cellular compartments. VP2 and VP4 expressed well only in the cytosol and no expression was attained for VP7 due to toxicity in host cells. Electron microscopic analysis of co-expressed VP2/6 and VP2/6/4 revealed assembled virus-like particles (VLPs) in the plant cytosol [44]. Yields of VP2/VP6 VLPs were as high as 1.1 g/kg of plant material, for batch sizes of around 100 g of leaves.

The potential of this product as a candidate vaccine is proven by a recent finding that VP2/VP6 VLPs made by conventional cell culture methods were a very effective priming vaccine for later boost by recombinant adenovirus expressing only VP6, in reducing virus shedding in challenged mice [45]. The commercial vaccines Rotarix® (GlaxoSmithKline) and Rotateq™ (Merck) target G1 P[8] and G1,2,3,4 and P[4] serotypes respectively. However, In South Africa, the predominant strains are the serotypes G8, G9 and G12 and P types [6], [8] and [9], and the commercial vaccines therefore have a considerably lower efficacy in this region [46]. Plant-made rotavirus G9 P[6] VLPs could therefore be a very useful addition in southern Africa to the conventional vaccines, especially if used in a prime-boost modality, in order to boost their efficacy.

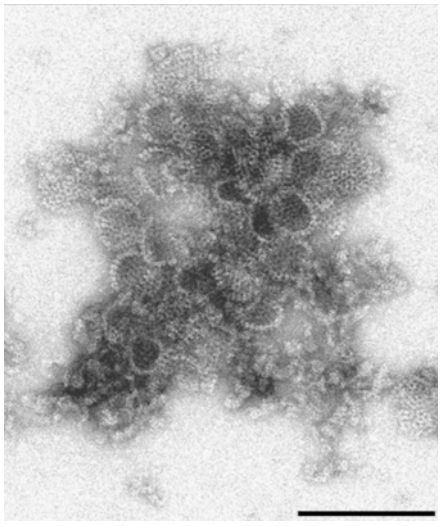

Fig. (3). Rotavirus VP2/6/4 co-expression in *N benthamiana:* protein extract partially purified by sucrose gradient centrifugation, particles captured onto electron microscope grids with mouse-anti VP6 antibody. Bar = 200 nm. From Mutepfa, 2011 [44].

University of Botswana

One of us (EPR) recently received a Masters dissertation to examine from the University of Botswana, that described using Tobacco mosaic virus (TMV) capsid protein fusions to display Foot

and mouth disease virus (FMDV) peptides as a potential FMDV vaccine [47] - which came as a complete surprise to someone who believed there were only two programmes in Africa on biofarming. This project derives from a new programme started at UB by Dr Larry Grill, one of the two founders of the now (sadly) defunct Large Scale Biology Corp. (Vacaville, Ca) and one of the original biofarming pioneers, who is now director of the Ferré/Marquet Vaccine Research Center at Pitzer College in Claremont, CA (http: //www.pitzer.edu/offices/vaccine_center/index.asp). The Center has been working since 2009 with UB researchers to create various vaccines: these include vaccines against rotavirus and FMDV, and a pilot project on lumpy skin disease, which is caused by a cattle-specific poxvirus. While there are commercial vaccines against lumpy skin disease, these are apparently not working very well in Botswana – and given that according to the Food and Agriculture Organisation (FAO), the disease is enzootic in most of sub-Saharan Africa and is an emerging disease threat to the Near and Middle East, it is a matter of some concern that it be controlled. The Botswana collaboration may be expanded, if funding is forthcoming, to include a GMP suite added onto existing facilities at the existing Botswana Vaccine Institute: this would allow processing of greenhouse-produced plant material in parallel with conventional activities, for final formulation and packaging alongside their other products (L Grill, pers. Comm.).

POTENTIAL FOR DEVELOPING COUNTRIES TO COMPETE IN BIOFARMING

The future of this sort of technology especially in developing countries, but also worldwide in the short term, may lie in exploiting niche opportunities. For example, it may be possible to provide high-grade pharmaceutical products at very low cost in resource-poor settings, to provide veterinary vaccines whose production is difficult and expensive (e.g.: BVDV, FMDV), there is a high burden of preventable disease (e.g.: rotavirus), or where there are no vaccines for "orphan diseases" [8].

Plant-made protein expression technology in these regions has great potential to address these needs, taking into account the proven existence of institutional scientific capacity to address the development of transgenic plants, and of human and veterinary vaccines.

The H5 candidate vaccine from the Cape Town group is an object example of the kind of potential in the developing world for biofarming, as it resulted from internal needs, was funded from internal sources, and used a completely local South African team. Essentially, the project owes its origin to a comment by a senior WHO person with an interest in influenza vaccines, who said in Cape Town at a local virology meeting in 2005 in a talk on H5N1 influenza, that *"…if a pandemic hits, you're on your own: no-one will send you vaccine*". Accordingly, EP Rybicki and A-L Williamson applied for an ad hoc grant from the local Poliomyelitis Research Foundation to explore the feasibility of making an emergency response vaccine to H5N1 or other type A influenza viruses. This was followed by a three-year grant from the same agency – in total, about US$250 000 over four years – and the project has since delivered two candidate plant-made haemagglutinin vaccines [42], a highly immunogenic DNA vaccine, and preliminary results on expression of HA from the 2009 H1N1pdm virus (EP Rybicki, A-L WIlliamson, E Mortimer, II Hitzeroth, S Mbewana, unpublished). Moreover, the group was able to get an important priority date on a patent application for the transient expression of H5 HA in plants [48]. This example is proof that developing countries can compete effectively in the larger biofarming arena – and with considerably less funding, and local interests at heart. As it happens, the 2009 H1N1 pandemic hit southern Africa with no imported vaccine being available for anyone but medical personnel until months after the local epidemic had peaked – pointing up the need for local on-demand manufacture for such vaccines.

While it is still a problem for developing country manufacturers to make human vaccines and therapeutics using plants due to a lack of appropriate facilities, the significant increase in recent years of dedicated clinical-grade manufacturing capacity using plant-made raw material may allow developing countries to bypass any such lack. For example, Kentucky BioProcessing (Owensboro, KY, USA) offers contract cGMP manufacture, which should greatly facilitate production of clinical lots of candidate vaccines or therapeutics. Other companies and institutions with in-house cGMP production facilities and experience with human clinical trials include Protalix Biotherapeutics (Carmel, Israel), Icon Genetics (Halle, Germany), The Fraunhofer Institute (Aachen, Germany), The Fraunhofer Center for Molecular Biotechnology (Newark, DE, USA) and Medicago Inc (Quebec City, Canada), who could possibly partner with other groups for clinical trial batches of biofarmed products. There is also the possibility of getting prefabricated cGMP or near-GMP facilities from a technology partner assembled on site in a developing country, for costs as low as US$5 million (R Chikwamba, CSIR, Pretoria, Pers. Comm.).

Although the biofarming research community has largely concentrated on how plant production could be used for human vaccines and therapeutics, it is even better suited to production of animal vaccines. The regulatory path for animal vaccines is far shorter and less rigorous than for human products, there is a shorter time to market, and quicker return on investment, and issues of side effects and even efficacy are less of a problem. Indeed, there have been successful proofs of efficacy of vaccines for Newcastle disease of chickens, foot and mouth disease, rabbit haemorrhagic disease and cottontail rabbit papillomavirus, by our groups among others, and USDA approval for release of the Newcastle disease vaccine by Dow AgroSciences LLC was obtained as long ago as 2006 [9].

The Botswana example is very illustrative of what could happen in the rest of Africa or other developing regions, given vision, a need for cheap veterinary vaccines, and a little bit of funding: in other words, application of established biofarming technologies to local problems, in the setting of an existing local facility providing quality veterinary vaccines. The technology also lends itself very well to "orphan" or "niche" vaccines, because of what is in effect infinite scalability of production – meaning it is well suited to production of animal vaccines, where "…the potential returns for animal vaccine producers are much less than those for human vaccines, with lower sales prices and smaller market sizes, resulting in a much lower investment in research and development in the animal vaccine area than in the human vaccine area, although the complexity and range of hosts and pathogens are greater." [49].

The range of hosts and pathogens is possibly nowhere greater than in Africa – with the added problems that most livestock farmers in Africa are resource-poor, and many of the diseases found here are not important in developed countries and are therefore not targeted by the big vaccine manufacturers. There are livestock vaccines produced in Africa – examples of manufacturers are the South African companies Onderstepoort Biological Products and Deltamune, the Botswana Vaccine Institute, the Laboratoire Central Vétérinaire, Mali, the Kenya Veterinary Vaccine Production Institute – and all of these facilities could benefit from the same model. That is, to include a GMP suite added onto existing facilities which would allow processing of greenhouse-produced plant material in parallel with conventional activities, for final formulation and packaging alongside other products. A similar model is presently being discussed with appropriate funders in South Africa, with the possibility of a modular pilot GMP-certified extraction facility being imported from the USA that would feed into existing vaccine manufacturing facilities. Products that could feed in soon could include avian influenza virus haemagglutinins, orbivirus virus-like particle vaccines, and possibly vaccine candidates for various African haemorrhagic fever viruses, for example.

On a wider scale, the Global Alliance for Livestock Veterinary Medicines (GALVmed; http: //www.galvmed.org), which is funded by the Bill & Melinda Gates Foundation, the UK Department for International Development and the European Commission, has its mission "*To make a sustainable difference in the access to veterinary medicines by poor livestock keepers in developing countries*". It has initially identified the African diseases East Coast fever and Rift Valley fever, as well as Newcastle disease and porcine cysticercosis, as its primary new vaccine targets; however, its mandate is global, and it expanded into Southern Asian countries during 2011, and will also potentially move into South American countries afflicted by extreme poverty, meaning the targeted animal diseases will change. GALVmed's approach is to more widely disseminate existing vaccines and the technology to make them - an approach that, although obviously effective, is a conservative one. Biofarming technology is well suited to "orphan" vaccines because of infinite scalability; add to this the benefit of much lower cost of material, and another means of fulfilling the stated mandate of a well-supported international NGO becomes clear. Possibly for the first time in modern vaccinology, we have a technology that allows the same means of production to be used at very different scale, depending on the size of the batch needed – without investing anything in stainless steel for fermentation or cell culture. For example, different-sized greenhouses could act as vaccine-specific production facilities, to feed into the same downstream processing unit and vialing and packaging facility – which could be the same one used by an established conventional facility, which would dramatically cut development costs.

It has not escaped our attention that establishment of such a model for veterinary vaccines would inevitably result in a cascade into human biologics – where, paradoxically, the developing world may be the region best suited to adopt it, given the vast pharmaceutical manufacturing capacity for generic vaccines and other in countries like India and Brazil. Indeed, Heber Biotec SA in Cuba (http: //www.heber-biotec.com/) has shown the way since 2006 by adopting a transgenic tobacco-made monoclonal antibody to HBsAg into its conventional yeast-made HBV vaccine manufacturing process: this has allowed the replacement of ascites fluid from 300 000 mice a year, and undoubtedly lowered costs [9]. It has also been used extensively inside and outside Cuba.

A very exciting recent development from South America has been the licencing by the Brazilian vaccines manufacturer Bio-Manguinhos of technology from the US company iBio (http: //ibioinc.com/), to produce a vaccine to yellow fever virus. The project will be a collaboration between iBio, Bio-Manguinhos and the Fraunhofer USA Center for Molecular Biology (FCMB), and will use the proprietary TMV-based "launch vector" technology [50]. If this collaboration gets products into a human vaccine pipeline, it will be a very significant development in global biofarming – and it will have started with a North-South partnership.

It is worth noting here that Bio-Manguinhos is a division of Fiocruz: this is the Fundação Oswaldo Cruz (Oswaldo Cruz Foundation, http: //www.ejolt.org/2011/09/fiocruz/), is attached to the Brazilian Ministry of Health, and is possibly the most prominent science and technology health institution in Latin America. Similarly, the Cuban Heber Biotec SA is a spinout of the government-funded Center for Genetic Engineering and Biotechnology (CIGB) in Havana (http: //www.cigb.edu.cu/). Both Bio-Manguinhos and Heber Biotec ship vaccines all over the world: the former produces yellow fever, poliomyelitis, DTP/Hib, measles/mumps/rubella and meningitis A and C vaccines; Heber exports vaccines for HBV, Hib, and combinations of DTP, Hib and HBV for infants. Thus, it is government-supported state vaccine manufacturers in developing countries that have been the most agile when it comes to adopting the biofarming technologies, and not the giant commercial entities of the developed North.

CONCLUDING REMARKS

The material presented above, as well as that covered in recent reviews, should convince that developing country institutions are more than capable of using biofarming technology for production of vaccines and therapeutics and other biological, of local and indeed of global relevance. However, development of established potential may be constrained by lack of funding, given a dearth of venture capital or established high-technology biotech firms in the global South.

Two of us – EP Rybicki and A Wigdorovitz – suggested at the 3rd Plant-Based Vaccines and Antibodies Conference in Verona in 2009 [7] that this could be remedied by partnerships between funding entities, institutions or companies in the developed "North", and groups such as ours in the under-developed notional South, which could be mutually advantageous in a number of ways. This is now expanded on here.

First, the cost of funding even sophisticated research would probably be significantly cheaper than in the North. While it is not a matter of pride that we generally get paid less than our northern counterparts, it is noteworthy that we in the less developed parts of the globe can probably get more value out of US dollar investments in research: research supplies and equipment cost only marginally more, buildings are probably far cheaper, and keeping laboratory animals is almost certainly less onerous. The increasing digitization of libraries, and preferential rates offered our libraries by large publishers, means we have access to the same scientific literature as everyone else – and we can almost certainly train students to a similar level of achievement for far less, given much lower costs of living in developing countries.

Second, legislation governing the use of transgenic organisms is often well established, and GM crops in particular are well entrenched: according to the most recent annual report of the International Service for the Acquisition of Agribiotech Applications (ISAAA; http: //www.isaaa.org/purchasepublications/ itemdescription.asp?ItemType=BRIEFS&Control=IB043-2011), developing countries are among the leading proponents of the use of GM plants – meaning there is no obvious barrier to use of the technology. Indeed, Ventria Bioscience has been growing transgenic rice in open fields in Argentina for some years for production of lactoferrin and lysozyme, intended as components of oral rehydration mixtures to combat diarrhoea [5].

Third, the regulatory and ethical frameworks in countries of the global South are often as sophisticated as those of the North: in fact, it is often possible to do ethical experiments using animals, and preclinical trials, to standards that are accepted by bodies such as the US National Institutes of Health. Moreover, South Africa in particular, but also countries like Thailand and Mexico, are very popular places to do large-scale clinical trials because of the well-established infrastructure and suitable populations.

Thus, the kind of partnering already pioneered between Brazil and a US company could become just the forerunner of an established trend: that is, Northern institutions and especially Big Pharma, investing in research centers in places with a sophisticated scientific work force like India, Brazil, Thailand, Malaysia, Argentina, South Africa and Cuba. They could tie up with prestigious local universities and research institutions, given that small biotechs are probably thin on the ground, and earn considerable credit for developing local potential, as well as pipelines of potential products. Who knows, it might even be possible to reduce drug and biological costs as a result, so that they can actually be used where they were researched. It would also help stop the inexorable brain drain from developing to developed countries, and – given the huge scale of the potential funding resource – go a long way to leveling the playing fields in pharmaceutical and biological research.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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BIBLIOGRAPHY

[1] Fooks AR. Development of oral vaccines for human use. Current opinion in molecular therapeutics 2000; 2(1): 80-6.

[2] Koprowski H, Yusibov V. The green revolution: plants as heterologous expression vectors. Vaccine 2001; 19(17-19): 2735-41.

[3] Walmsley AM, Arntzen CJ. Plants for delivery of edible vaccines. Current opinion in biotechnology 2000; 11(2): 126-9.

[4] Yu J, Langridge WH. Novel Approaches to Oral Vaccines: Delivery of Antigens by Edible Plants. Curr Infectious Disease Reports 2000; 2(1): 73-77.

[5] Rybicki EP. Plant-produced vaccines: promise and reality. Drug Discovery Today 2009; 14(1-2): 16-24.

[6] FDA. FDA approves new orphan drug to treat a form of Gaucher disease. (2012).

[7] Rybicki EP. Third International Conference on Plant-Based Vaccines and Antibodies. Expert review of vaccines 2009; 8(9): 1151-5.

[8] Rybicki EP, Chikwamba R, Koch M, Rhodes JI, Groenewald JH. Plant-made therapeutics: an emerging platform in South Africa. Biotechnol Adv 2012; 30(2): 449-59.

[9] Rybicki EP. Plant-made vaccines for humans and animals. Plant Biotechnology Journal 2010; 8(5): 620-37.

[10] Penney CA, Thomas DR, Deen SS, Walmsley AM. Plant-made vaccines in support of the Millennium Development Goals. Plant cell reports 2011; 30(5): 789-98.

[11] Pérez Aguirreburualde MS, Gómez MC, Ostachuk A, *et al*. Efficacy of a BVDV subunit vaccine produced in alfalfa transgenic plants. Efficacy of a BVDV subunit vaccine produced in alfalfa transgenic plants (2012).

[12] Lentz EM, Mozgovoj MV, Bellido D, Dus Santos MJ, Wigdorovitz A, Bravo-Almonacid FF. VP8* antigen produced in tobacco transplastomic plants confers protection against bovine rotavirus infection in a suckling mouse model. J biotechnology 2011; 156(2): 100-7.

[13] Rybicki EP, Martin DP. Virus-Derived ssDNA Vectors for the Expression of Foreign Proteins in Plants. Current topics in microbiology and immunology (In Press)(2013).

[14] Werner S, Breus O, Symonenko Y, Marillonnet S, Gleba Y. High-level recombinant protein expression in transgenic plants by using a double-inducible viral vector. Proc Natl Acad USA 2011; 108(34): 14061-6.

[15] Carrillo C, Wigdorovitz A, Oliveros JC *et al*. Protective immune response to foot-and-mouth disease virus with VP1 expressed in transgenic plants. J Virol 1998; 72(2): 1688-90.

[16] Carrillo C, Wigdorovitz A, Trono K *et al*. Induction of a virus-specific antibody response to foot and mouth disease virus using the structural protein VP1 expressed in transgenic potato plants. Viral immunology 2001; 14(1): 49-57.

[17] Wigdorovitz A, Perez Filgueira DM, Robertson N *et al*. Protection of mice against challenge with foot and mouth disease virus (FMDV) by immunization with foliar extracts from plants infected with recombinant tobacco mosaic virus expressing the FMDV structural protein VP1. Virology 1999; 264(1): 85-91.

[18] Saiz JC, Cairo J, Medina M *et al*. Unprocessed foot-and-mouth disease virus capsid precursor displays discontinuous epitopes involved in viral neutralization. J Virol 1994; 68(7): 4557-64.

[19] Mayr GA, Chinsangaram J, Grubman MJ. Development of replication-defective adenovirus serotype 5 containing the capsid and 3C protease coding regions of foot-and-mouth disease virus as a vaccine candidate. Virology 1999; 263(2): 496-506.

[20] Dus Santos MJ, Carrillo C, Ardila F *et al*. Development of transgenic alfalfa plants containing the foot and mouth disease virus structural polyprotein gene P1 and its utilization as an experimental immunogen. Vaccine 2005; 23(15): 1838-43.

[21] Dus Santos MJ, Wigdorovitz A, Trono K *et al*. A novel methodology to develop a foot and mouth disease virus (FMDV) peptide-based vaccine in transgenic plants. Vaccine 2002; 20(7-8): 1141-7.

[22] Wigdorovitz A, Mozgovoj M, Santos MJ *et al*. Protective lactogenic immunity conferred by an edible peptide vaccine to bovine rotavirus produced in transgenic plants. J General Virol 2004; 85(Pt 7): 1825-32.

[23] Perez Filgueira DM, Mozgovoj M, Wigdorovitz A *et al*. Passive protection to bovine rotavirus (BRV) infection induced by a BRV VP8* produced in plants using a TMV-based vector. Archives Virol 2004; 149(12): 2337-48.

[24] Perez Filgueira DM, Zamorano PI, Dominguez MG *et al*. Bovine herpes virus gD protein produced in plants using a recombinant tobacco mosaic virus (TMV) vector possesses authentic antigenicity. Vaccine 2003; 21(27-30): 4201-9.

[25] Pérez Aguirreburualde MS, Gomez MC, Ostachuk A *et al*. Efficacy of a BVDV subunit vaccine produced in alfalfa transgenic plants. Veterinary immunology and immunopathology 2013; 151(3-4): 315-24.

[26] Wirth S, Segretin ME, Mentaberry A, Bravo-Almonacid F. Accumulation of hEGF and hEGF-fusion proteins in chloroplast-transformed tobacco plants is higher in the dark than in the light. J Biotechno 2006; 125(2): 159-72.

[27] Lentz EM, Segretin ME, Morgenfeld MM *et al*. High expression level of a foot and mouth disease virus epitope in tobacco transplastomic plants. Planta 2010; 231(2): 387-95.

[28] Lentz EM, Garaicoechea L, Alfano EF, Parreno V, Wigdorovitz A, Bravo-Almonacid FF. Translational fusion and redirection to thylakoid lumen as strategies to improve the accumulation of a camelid antibody fragment in transplastomic tobacco. Planta 2012; 236(2): 703-14.

[29] Zelada AM, Calamante G, De La Paz Santangelo M *et al*. Expression of tuberculosis antigen ESAT-6 in Nicotiana tabacum using a potato virus X-based vector. Tuberculosis 2006; 86(3-4): 263-7.

[30] Wigdorovitz A, Carrillo C, Dus Santos MJ *et al*. Induction of a protective antibody response to foot and mouth disease virus in mice following oral or parenteral immunization with alfalfa transgenic plants expressing the viral structural protein VP1. Virology 1999; 255(2): 347-53.

[31] Berinstein A, Vazquez-Rovere C, Asurmendi S *et al*. Mucosal and systemic immunization elicited by Newcastle disease virus (NDV) transgenic plants as antigens. Vaccine 2005; 23(48-49): 5583-9.

[32] Clemente M, Curilovic R, Sassone A, Zelada A, Angel SO, Mentaberry AN. Production of the main surface antigen of Toxoplasma gondii in tobacco leaves and analysis of its antigenicity and immunogenicity. Mol Biotechnol 2005; 30(1): 41-50.

[33] Ferraro G, Becher ML, Angel SO, Zelada A, Mentaberry AN, Clemente M. Efficient expression of a Toxoplasma gondii dense granule Gra4 antigen in tobacco leaves. Experimental Parasitol 2008; 120(1): 118-22.

[34] Gomez E, Zoth SC, Asurmendi S, Vazquez Rovere C, Berinstein A. Expression of hemagglutinin-neuraminidase glycoprotein of newcastle disease Virus in agroinfiltrated Nicotiana benthamiana plants. J Biotechnol 2009; 144(4): 337-40.

[35] Nelson G, Marconi P, Periolo O, La Torre J, Alvarez MA. Immunocompetent truncated E2 glycoprotein of bovine viral diarrhea virus (BVDV) expressed in Nicotiana tabacum plants: a candidate antigen for new generation of veterinary vaccines. Vaccine 2012; 30(30): 4499-504.

[36] Rybicki EP. Agricultural molecular biotechnology in South Africa: new developments from an old industry. Agricultural molecular biotechnology in South Africa: new developments from an old industry 2013(30/1/13)(1999).

[37] Rybicki EP, Williamson AL, Meyers A, Hitzeroth, Ii. Vaccine farming in Cape Town. Human Vaccines 2011; 7(3).

[38] Sainsbury F, Lomonossoff GP. Extremely high-level and rapid transient protein production in plants without the use of viral replication. Plant Physiol 2008; 148(3): 1212-8.

[39] Varsani A, Williamson AL, Rose RC, Jaffer M, Rybicki EP. Expression of Human papillomavirus type 16 major capsid protein in transgenic Nicotiana tabacum cv. Xanthi. Archives of Virol 2003; 148(9): 1771-86.

[40] Giorgi C, Franconi R, Rybicki EP. Human papillomavirus vaccines in plants. Expert Review of Vaccines 2010; 9(8): 913-24.

[41] Kane MA. Preventing cancer with vaccines: progress in the global control of cancer. Cancer Prevention Res 2012; 5(1): 24-29.

[42] Mortimer E, Maclean JM, Mbewana S *et al*. Setting up a platform for plant-based influenza virus vaccine production in South Africa. BMC Biotechnology 2012; 12: 14.

[43] Landry N, Ward BJ, Trepanier S *et al*. Preclinical and clinical development of plant-made virus-like particle vaccine against avian H5N1 influenza. PloS one 2010; 5(12): e15559.

[44] Mutepfa DL. Expression of rotavirus capsid proteins in N. benthamiana leaves using an agrobacterium-mediated transient expression system. Master of Science Dissertation, University of Cape Town, 97pp (2011).

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[45] Zhou H, Guo L, Wang M *et al*. Prime immunization with rotavirus VLP 2/6 followed by boosting with an adenovirus expressing VP6 induces protective immunization against rotavirus in mice. Virol J 2011; 8, 3.

[46] Seheri LM, Page NA, Mawela MP, Mphahlele MJ, Steele AD. Rotavirus vaccination within the South African Expanded Programme on Immunisation. Vaccine 2012; 30 Suppl 3, C14-20.

[47] Phiri KB. Agro-infiltration mediated expression of FMDV SAT-1 and SAT-2 P1 epitopes from Botswana isolates as surface presented antigens in TMV coat protein fusions. Master of Science Dissertation, University of Botswana. 97pp. (2011).

[48] Williamson A-L, Rybicki EP, Maclean JM, Becker-Hitzeroth II. Expression of viral proteins in plants. WO 2006/119516 (2006).

[49] Meeusen EN, Walker J, Peters A, Pastoret PP, Jungersen G. Current status of veterinary vaccines. Clinical Microbiol Rev 2007; 20(3): 489-510, table of contents.

[50] Musiychuk K, Stephenson N, Bi H *et al*. A launch vector for the production of vaccine antigens in plants. Influenza and other respiratory viruses 2007; 1(1): 19-25.