Pharmaceutical Proteins in Plants

A Strategic Genetic Engineering Approach for the Production of Tuberculosis Antigens

Roger Frutos,[a] Hubert Denise,[b] Christian Vivares,[b] Jean-Marc Neuhaus,[c] Sandro Vitale,[d] Emmanuela Pedrazzini,[d] Julian Ma,[e] Phil Dix,[f] John Gray,[g] Mario Pezzotti,[h] Udo Conrad,[i] and David Robinson[j]

[a]French Agricultural Research Center for International Development, Campus International de Baillarguet, Montpellier, France

[b]Université Blaise Pascal Clermont-Ferrand II, Aubière, France

[c]Université de Neuchâtel, Neuchâtel, Switzerland

[d]Istituto di Biologia e di Biotecnologia Agraria, Milano, Italy

[e]St. George's Hospital, London, United Kingdom

[f]National University of Ireland, Maynooth, Ireland

[g]University of Cambridge, Cambridge, United Kingdom

[h]Universita di Verona, Verona, Italy

[i]Universität Gatersleben, Gatersleben, Germany

[j]Universität Heidelberg, Heidelberg, Germany

Tuberculosis (TB) is a re-emerging disease that is considered a major human health priority as well as an important disease of livestock. TB is also a zoonosis, and *Mycobacterium tuberculosis* and *M. bovis*, the human and bovine causative agents, respectively, are very closely related. Protection against TB is essentially achieved through vaccination with the Bacille Calmetle-Guerin (BCG) strain of *M. bovis*. Protection is, however, incomplete, and novel improved vaccines are currently under investigation. Production of protective antigens in transgenic plants, or "pharming," is a promising emerging approach, and a zoonosis-like TB is a good model for investigating the potential of this approach. Pharma-Planta, a European Commission-funded project and consortium, was set up to address this topic, within which a component is aimed at assessing the production efficacy and stability of the TB antigens in different compartments of the plant cell. This article is meant to introduce this promising approach for veterinary medicine by describing the ongoing project and its specific genetic engineering strategy.

Key words: **tuberculosis; plant biotechnology; vaccines; pharming; Pharma-Planta**

Introduction

Tuberculosis (TB) is a re-emerging disease which infects one-third of the world population, resulting annually in 9 million new cases and 3 million deaths.[1,2] TB is also an important animal disease resulting in significant losses. Protection against TB is essentially achieved through vaccination with the Bacille Calmetle-Guerin (BCG) strain of *Mycobacterium bovis*. However, BCG confers limited protection,[1] interferes with skin tests for diagnostic and epidemiological survey,[3] and is highly susceptible to environmental constraints, such as cold chain interruption. Novel

Address for correspondence: Roger Frutos, UMR 17, Cirad, TA A17, Campus International de Baillarguet, 34398 Montpellier cedex 5, France. Voice: +33 (0) 4 67 59 39 62; fax: +33 (0) 4 67 59 37 98. roger.frutos@cirad.fr

Animal Biodiversity and Emerging Diseases: Ann. N.Y. Acad. Sci. 1149: 275–280 (2008).
doi: 10.1196/annals.1428.089

vaccines are thus currently under investigation. Production of protective antigens in transgenic plants is one of these exploratory approaches. Plant production of pharmaceuticals is an emerging field with high efficiency in production of antibodies.[4] However, production of antigens is more delicate owing to lower expression levels, influence of cell compartments in stability, and presence of plant contaminants, all resulting in low purification yields. This article summarizes genetic engineering approaches specifically designed to investigate the influence of each cell compartment in the production efficiency of an antigen. This project is a part of the European Commission-funded Pharma-Planta project, aimed at developing and mastering production of pharmaceuticals in plants and focusing on TB as a model.

The Pharma-Planta Project

The Pharma-Planta project is a consortium of 39 principal scientists from academic and industrial institutions in Europe and South Africa. Pharma-Planta is funded by the EC and aims to build a plant-based production platform for pharmaceuticals in Europe and to enter the first candidate pharmaceuticals into human clinical trials. Plants have enormous potential for the production of recombinant pharmaceutical proteins as they are inexpensive, versatile, and amenable to rapid and economical scale-up. The project addresses pharmaceuticals for the prevention of HIV, rabies, TB, and diabetes that remain significant health problems both in Europe and the developing world.

A Promising Approach for Future Generations of Vaccines

Production of pharmaceuticals in plants is definitely a promising approach and is already capable of delivering large amounts of high-purity antibodies at a very competitive cost. The production of specific high-added value, such as lipase or insulin, is also successfully implemented.[5,6] However, production of antigens, and thus vaccines, is still in the exploratory track owing to high variability of expression efficiency, depending both upon cell compartments and foreign protein. The purification of antigens from plants is the first step toward the production of edible vaccines in plants, which could definitely lower the cost of vaccination for many diseases. This approach is of particular interest in veterinary medicine considering the cost of vaccination with respect to limited added value of the products. However, there is as yet no way to predict the production efficiency of antigens in plants, and, should this approach prove valuable for vaccine production, there is currently a need for comprehensive investigation on the potential of each cell compartment for the production of antigens.

A Specific Genetic Engineering Approach

To investigate the respective influence of each cell compartment on protein stability, production, and recovery efficiency, a single transgene cassette was expressed in *Nicotiana tabaccum* var. *petit havanna*, a variety of tobacco that was selected for comparative expression analysis, and the following cell compartments of the same transgene cassette: chloroplast, cytoplasm, cytoplasm anchored to the cytosolic side of the reticulum membrane, endoplasmic reticulum anchored to the lumen side, Golgi accumulation in the lumen, Golgi anchored to the membrane on the lumen side, and plasma membrane (Fig. 1, Table 1). Furthermore, additional strategies were considered to favor accumulation of the Esat-6/Ag85b protein and thus specific enrichment, including accumulation of the target protein in the lumen of the endoplasmic reticulum by fusion with a zein domain, enrichment and improved purification by fusion

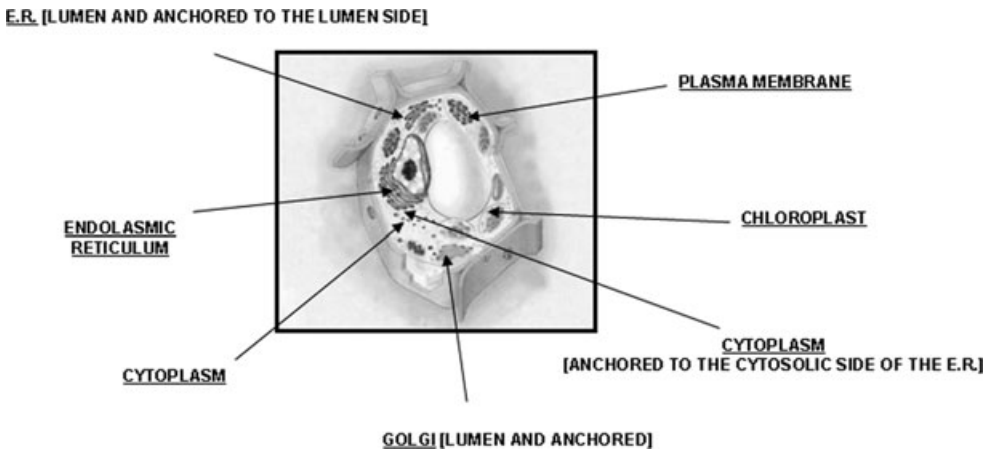

Figure 1. Target expression of the fusion protein in plant cell compartments. E.R., endoplasmic reticulum; IgA, Immunoglobulin A.

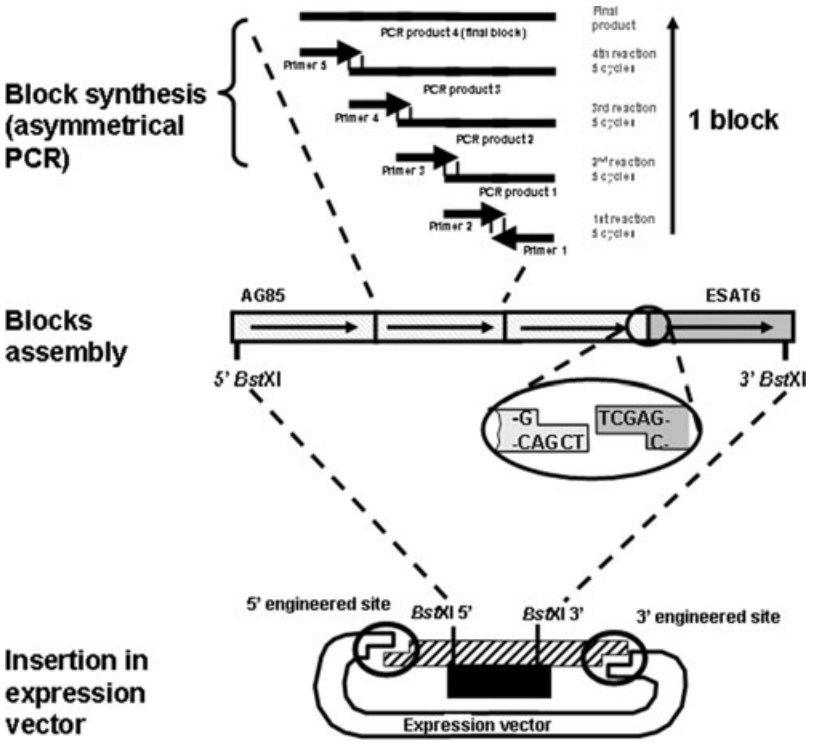

Figure 2. Strategy for *in vitro* creation of the optimized synthetic fusion gene. Synthetic optimized genes are created *in vitro* by recursive asymmetric PCR by annealing first long primers 1 and 2, which are complementary over 15 nucleotides, and doing five cycles of PCR. The first PCR product is, in turn, mixed with long primer 3, which also overlaps for 15 nucleotides, to yield PCR product after five cycles. This is repeated until addition of long primer 5. The final PCR product is cloned and sequenced prior to assembly with the other four blocks, using restriction sites specifically engineered for that purpose. The full-length synthetic gene is, in turn, sequenced and inserted in the various expression vectors using specific adapters.

TABLE 1. Specific Components for Cell Compartment Targeting

System	Promoter	Targeting element	Objective	Reference
Chloroplast expression	rrn promoter	Chloroplast transformation vector pZS197-JH1 Chloroplast rbcL ribosome binding site aadA selectable marker	Purification	15
Cytoplasmic production	35S promoter Gelvin "superpromoter"	None	Control reference	16
Anchoring to cytosolic side of ER	Gelvin "superpromoter"	Cyt. B5 transmembrane domain 19 aa OP3 site	Purification	17
Transmembrane domain I (ER)	35S promoter	Lamp1 transmembrane domain TM17	Purification	18
Transmembrane domain II (golgi)	35S promoter	Lamp1 transmembrane domain TM20	Purification	18
Transmembrane domain II (plasma membrane)	35S promoter	Lamp1 transmembrane domain TM23	Purification	18
Elastin-like peptide fusion	35S promoter	ELP fusion peptide KDEL ER retention signal	Enrichment	19
β-zein fusion	35S promoter	Zein signal peptide Zein domain	Enrichment	20
Antibody–antigen fusion	35S promoter	IgA α2-α3 domains	Enrichment	21,22

with an elastin polypeptide, and enrichment and improved purification of the fusion protein by fusion with an antibody (Table 1).

A Single Synthetic Transgene Cassette Targeted to Several Compartments

The selected transgene cassette is a fusion gene comprising two genes *Esat-6* and *ag85b*[5] from *M. tuberculosis* and *M. bovis*. Early works showed that effective vaccination could be achieved with this fusion protein.[7–9] Expression of bacterial genes in plants often requires first modifying and adapting the coding sequence to that of the plant.[10] A synthetic optimized gene was thus created *in vitro* by recursive asymmetric PCR method.[11–14] The synthetic *Esat-6/ag85b* fusion gene was assembled from four individual PCR-created blocks of 267 nucleotides (nt),

309 nt, 314 nt, and 314 nt, respectively (Fig. 2). Targeting the protein to the various compartments was achieved through the insertion of transmembrane domains, targeting domains or specific recombination vectors.

Concluding Remarks

Transient expression was achieved for all constructs, and stable expression is currently underway. However, owing to the time required for stable plant transformation, line selection, and selfing, no production can be expected before an additional year. The availability of stable transgenic lines will allow for final comparative assessment of the potential of each compartment for the production of the same antigen. Furthermore, the additional enrichment strategies developed here will also provide information on the potential for increased yield. Such research projects are long-term projects requiring numerous subsequent steps, and we are still far from the final outcome. However, it was essential to introduce this novel approach to the biannual Society for Tropical Veterinary Medicine meeting owing to its important potential for human and animal health. It is also a way to fill the gap between plant and animal biotechnology and exemplify how a global approach can be beneficial to everyone. Finally, it is, to our knowledge, the first time such a comprehensive genetic engineering strategy was developed to assess the influence of plant cell compartments on expression of pharmaceutical proteins.

Conflicts of Interest

The authors declare no conflicts of interest.

References

1. Britton, W.J. & U. Palendira. 2003. Improving vaccines against tuberculosis. *Immunol. Cell Biol.* **8:** 34–45.
2. Dye, C. *et al*. 1999. Consensus statement. Global burden of tuberculosis: estimated incidence, prevalence and mortality by country. WHO Global Surveillance and Monitoring Project. *JAMA* **282:** 677–686.
3. Lowrie, D.B. *et al*. 1995. Vaccination against tuberculosis. *Int. Arch. Allergy Immunol.* **108:** 309–312.
4. Stoger, E. *et al*. 2005. Sowing the seeds of success: pharmaceutical proteins from plants. *Curr. Opin. Biotechnol.* **16:** 167–173.
5. Nykiforuk *et al*. 2006. Transgenic expression and recovery of biologically active recombinant human insulin from *Arabidopsis thaliana* seeds. *Plant Biotechnol.* **4:** 77–85.
6. Spok, A. 2006. Molecular farming on the rise—GMO regulators still walking a tightrope. *Trends Biotechnol.* **25:** 74–82.
7. Derrick, S.C. *et al*. 2004. A polyvalent DNA vaccine expressing an ESAT6-Ag85B fusion protein protects mice against a primary infection with *Mycobacterium tuberculosis* and boosts BCG-induced protective immunity. *Vaccine* **23:** 780–788.
8. Olsen, A.W. *et al*. 2001. Protection of mice with a tuberculosis subunit vaccine based on a fusion protein of antigen 85b and esat-6. *Infect. Immun.* **69:** 2773–2778.
9. Olsen, A.W. *et al*. 2004. Protective effect of a tuberculosis subunit vaccine based on a fusion of antigen 85B and ESAT-6 in the aerosol guinea pig model. *Infect. Immun.* **72:** 6148–6150.
10. Perlak, F.J. *et al*. 1991. Modification of the coding sequence enhances plant expression of insect control protein genes. *Proc. Natl. Acad. Sci. USA* **88:** 3324–3328.
11. Promodrou, C. & L.H. Pearl. 1992. Recursive PCR: a novel technique for total gene synthesis. *Prot. Eng.* **5:** 827–829.
12. Leroy, T. *et al*. 2000. *Agrobacterium*-mediated transformation of *Coffea* sp. using the *cry1Ac* gene of *Bacillus thuringiensis*. *Plant Cell Rep.* **19:** 382–385.
13. Breitler, J.C. *et al*. 2000. Expression of a *Bacillus thuringiensis cry1B* synthetic gene protects Mediterranean rice against the striped stem borer. *Plant Cell Rep.* **19:** 1195–1202.
14. Bohorova, N. *et al*. 2001. Novel synthetic *Bacillus thuringiensis cry1B* gene and the *cry1B-cry1Ab* translational fusion confer resistance to southwestern corn borer, sugarcane borer and fall armyworm in transgenic tropical maize. *Theor. Appl. Genet.* **103:** 817–826.
15. Maggio, C. *et al*. 2007. Intracellular sorting of the tail-anchored protein cytochrome b5 in plants: a comparative study using different isoforms from rabbit and *Arabidopsis*. *J. Exp. Biol.* **6:** 1365–1379.
16. Birch-Machin, I. *et al*. 2004. Accumulation of rotavirus VP6 protein in chloroplasts of transplastomic tobacco is limited by protein stability. *Plant Biotech. J.* **2:** 261–270.

17. Avesani, L. *et al*. 2003. Improved in planta expression of the human islet autoantigen glutamic acid decarboxylase (GAD35). *Transgenic Res.* **12:** 203–217.
18. Brandizzi, F. *et al*. 2002. The destination for single-pass membrane proteins is influenced by the length of the hydrophobic domain. *Plant Cell Rep.* **14:** 1077–1092.
19. Lin, M. *et al*. 2006. Functional expression of a biologically active fragment of soluble gp130 an an ELP-fusion protein in transgenic plants: purification via inverse transition cycling. *Biochem. J.* **398:** 577–583.
20. Mainieri, D. *et al*. 2004. Zeolin. A new recombinant storage protein constructed using g-zein and bean phaseolin. *Plant Physiol.* **136:** 3447–3456.
21. Charlelegue, D. *et al*. 2005. Highly immunogenic and protective recombinant vaccine candidate expressed in transgenic plants. *Infect. Immun.* **73:** 5913–5922.
22. Obregon, P. *et al*. 2006. HIV-1 p24-immunoglobulin fusion molecule: a new strategy for plant-based protein production. *Plant Biotechnol. J.* **4:** 195–207.