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GM PLANTS AS BIOFACTORIES OF PHARMACEUTICAL PROTEINS: PRESENT STATE AND FUTURE DEVELOPMENTS

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Abstract

The use of genetically modified organisms (GMOs) as biofactories for the production of recombinant proteins of commercial interest is at present one of the major applications of 'molecular' biotechnology, and the business basis of many modern biotech companies. Since marketing in the early 1980s of recombinant human insulin, synthesised in Escherichia coli, hundreds of proteins with pharmacological activity, used for the diagnosis, treatment of prevention of human (and animal) diseases, have been produced in different platforms. The intrinsic limitations of bacterial cell cultures – especially the lack of the machinery for post-translanslational modifications of proteins, which are required for the synthesis of pharmacologically active proteins – have made mammalian cell cultures the system of choice for the industrial production of biopharmaceuticals. These are robust, reliable and highly controlled production systems, optimised over the years and for which GMP ('good manufacturing practice') procedures have been established and approved by the competent authorities. Mammalian cell cultures, however, have also important limitations and drawbacks, mostly regarding their high costs, relatively low productivity and lack of flexibility to scale-up or -down the production, in response to market demands. Many of these limitations could be overcome with the use of plant biofactories, the so-called '3rd generation' of genetically modified plants used for the commercial production of recombinant proteins including, more specifically, pharmaceutical proteins: 'molecular pharming' or 'pharma crops. However, despite the important advantages – at least theoretically – of GM plants, development of this kind of production platforms has been slow and the first plant-made biopharmaceuticals have been approved for human use only recently. This has been due mostly to regulatory issues rather than to scientific or technical problems, but recent developments indicate a rapid growth of this technology, even if it is limited to niche markets for specific plant-made protein drugs.

Key words*: biopharmaceuticals, cell cultures, molecular 'pharming', recombinant proteins, transgenic plants.*

INTRODUCTION

The *in vitro* construction, for the first time, of biologically functional bacterial plasmids conferring antibiotic resistance when transformed into *Escherichia coli* (Cohen et al., 1973) marked the birth of the 'recombinat DNA technology' or 'genetic engineering', starting a revolution in the experimental strategies used to investigate biological processes. In addition, from a practical point of view, this new biotechnology opened the possibility to produce large amounts of any protein, by expression of the corresponding gene in a suitable 'genetically modified organism' (GMO), once that gene had been cloned in an appropriate vector. In 1976, Herbert Boyer – one of the authors of the 1973 seminal paper – and venture capitalist Robert Swanson, founded Genetech, the first modern biotech company, devoted to the commercial production of pharmaceutical products using recombinant DNA technology. Human insulin synthesised in *E. coli* was the first recombinant therapeutic protein to reach the market, in 1982 (produced under licence by Eli Lilly & Co.), followed by recombinant human growth hormone, in this case made directly by Genetech, which was approved by the FDA in 1985.

Since then, different GMO systems have been used as 'biofactories' for the synthesis of a wide range of proteins with pharmacological activities, such as hormones, growth factors, blood clotting factors, antibodies, enzymes or vaccines, and many other proteins with

application in the diagnosis, prevention or treatment of human (and animal) diseases. Bacterial cultures are the simplest platforms for commercial production of recombinant proteins, but their intrinsic characteristics, regarding the capacity to synthesise pharmaceutically active products, have limited their use to the synthesis of a reduced subset of small and simple proteins that do not require post-translational modifications for their biological activity. At present, *in vitro* cultures of mammalian cells are the system of choice for the industrial production of commercial biopharmaceuticals, despite their technical complexity and high costs. The use the so-called '3rd generation' of transgenic plants as biofactories for the production of recombinant proteins for the pharmaceutical industry has been proposed a long time ago, after efficient methods for plant genetic transformation were establishes in the late 1980s. Despite a number of theoretical advantages of plant-based platforms, as compared to bacterial and mammalian cell cultures – and also in relation to the production of recombinant proteins in the milk of transgenic mammals – development of commercial 'pharma crops' is lagging far behind fermenter-based systems. In this short review, we will briefly describe the main characteristics of the different production platforms, with their pros and cons, the advantages of GM plants-based systems and the reasons why their potential has not yet been realised. We will also discuss some recent developments which point to a wider use of transgenic plants for the production of biopharmaceuticals in the near future, at least for specific applications.

BACTERIA AND YEAST CULTURES

In vitro cultures of bacterial cells – of *E. coli*, in most cases – show several advantages for the production of recombinant proteins, being simple, reliable and relatively cheap systems, easy to establish and maintain, since there is a vast knowledge on the procedures for bacteria genetic manipulation. Bacteria grow very fast, to a high cell density in the cultures, which present high protein production levels. These systems are well-established, have been used for many years, and do not pose regulatory problems.

Bacterial cultures have also serious drawbacks, especially for the expression of complex and multimeric proteins, which in many cases are not produced in an active form in the cells, since they do not fold or are not assembled properly. However, their most important limitation is that the bacteria do not possess the machinery responsible for post-translational modification of proteins. Most human proteins are modified by phosphorylation, acetylation, glycosylation, etc., and the presence of these groups, especially a correct glycosylation, is essential for their biological (and consequently pharmacological) activity. Therefore, this platform can only be used in practice for the synthesis of simple, generally small proteins that do not require glycosylation or other post-translational modifications for their activity (e.g, some polypeptide hormones such as insulin, growth hormone, parathyroid hormone or calcitonin).

Yeast cell cultures, mostly of *Sacharomyces cerevisiae* and more recently of *Pichia pastoris*, are also used for the production of recombinant proteins. They have characteristics similar to those of bacterial cells, both regarding advantages and limitations. Although they are eukaryotic cells, in which proteins can be post-translationally modified, the patterns of protein glycosylation, are different from those of mammalian cells. Therefore, the range of recombinant human proteins that can be synthesised in yeast in a pharmacologically active form is also similar to bacteria.

MAMMALIAN CELL CULTURES

In vitro cultures of mammalian cells are also very robust, highly controlled and reliable systems. There is a long experience in their industrial use as biofactories, and a whole body of 'good manufacturing practices' (GMP) has been developed over the years, with approval by the competent regulatory authorities. Unlike bacteria, however, they ensure (in general) the synthesis of pharmacologically active products, since post-translational modifications of the recombinant protein are the same or very similar than that of the native human protein *in vivo*.

That is why mammalian cell cultures – for example, of Chinese Hamster Ovary (CHO)

cells, the 'golden standard' in the industry – are currently the system of choice for the production of biopharmaceuticals, despite having several drawbacks and limitations:

i) very complex systems, with high costs of both, up-front investment and maintenance (high-tech, high-cost);

ii) slow growth of the cells and limited productivity;

iii) the difficulty (technical and economic) to scale up or down production;

iv) the risk of contamination by human pathogens (e.g., viruses or prions).

GENETICALLY MODIFIED (GM) PLANTS: MOLECULAR 'PHARMING'

Production of recombinant proteins in the so-called '3rd generation' of transgenic plants provides an alternative, or rather a complement, to fermenter-based systems of genetically modified microorganisms or mammalian cells in *in vitro* cultures. The term 'molecular farming' was coined to describe the use of GM plants as biofactories of recombinant proteins, in general, and 'molecular *pharming*' or 'pharma crops' if we refer specifically to pharmacological proteins. These plant-based systems have – at least theoretically – a number of important advantages over other commercial production platforms:

i) the methods of plant genetic transformation – mediated by *Agrobacterium tumefaciens* (Herrera-Estrella et al., 1983) or by microprojectile bombardment (Klein et al., 1987) – and regeneration of transgenic plants are relatively cheap and simple, as compared for example to the generation of transgenic animals.

ii) the production systems can be established with low up-front investment and maintained cheaply, since they are based on common techniques used for centuries in agriculture (low-tech, low-cost).

iii) the production can be scaled (up or down) easily and cheaply, to adapt to market demands (in principle, simply by increasing or decreasing the cultivation area).

iv) in general, proteins are synthesised in a pharmacologically active form, since the systems of post-translational modification (e.g. glycosylation) in plants are similar to those of mammalian cells.

v) the synthesis of the recombinant protein can be directed to specific organs (and organelles) by using tissue-specific promoters and proper subcellular localization signals. Thus, the protein can be 'encapsulated' in natural plant structures, for example in the endosperm of seeds, facilitating in this way the storage of the protein in an active form, without requiring special conditions such as refrigeration

vi) there is the possibility of developing simple and efficient purification methods.

vii) there is no risk of contamination with human pathogens.

TRANSIENT EXPRESSION SYSTEMS

Besides generation of stably transformed GM plants, protocols have been established for the transient expression of the recombinant proteins in plant tissues, tobacco and alfalfa leaves being the commonest targets. Different vectors can be used to deliver cloned DNA: *Agrobacterium tumefaciens* (by vacuum-infiltration in the leaves), recombinant plant viruses or hybrid vectors containing viral sequences delivered by *Agrobacterium*. These transient expression systems allow the rapid production of large quantities of biopharmaceuticals, which would be needed to treat large numbers of individuals in a short period of time in case, for example, of epidemics or bioterrorist attacks.

EDIBLE VACCINES

Another specific application of 'pharma-crops' is the low-cost oral delivery of protein drugs bioencapsulated in plant cells (Kwon and Daniell, 2015). Among all these plant-derived recombinant proteins, especial attention has been given to the production of edible vaccines, to replace 'traditional' vaccines (consisting in live attenuated viruses or bacteria, killed or inactivated bacteria, or specific surface antigens of pathogens). Ingesting plant material containing a suitable recombinant antigen will induce an immune response at the level of the intestinal mucosa, via gut-associated lymphoid tissues. The natural 'encapsulation' of the recombinant vaccine in plant cells or subcellular structures (plastids, protein bodies, etc.), apart from protecting the protein from

acid and enzymes in the stomach, may help activate the immune system without the need of an adjuvant. Accordingly, a variety of vaccine targets – surface antigens of different viruses, *E. coli* and cholera toxins, etc. – have been expressed in edible transgenic plants, such as maize, tomato, lettuce, spinach or rice, and some of these products are undergoing clinical trials, previous to approval and commercial-lisation.

This approach would reduce many of the problems associated with the production and application of traditional vaccines, such as the high cost of vaccine purification, as well as transport and distribution issues (most impor-tant in developing countries): no need to maintain the 'cold chain', avoiding risk of transmitting infections by the use of non-sterile syringes, etc.

SOME EXAMPLES OF RECOMBINANT PROTEINS EXPRESSED IN GM PLANTS

Since the early 1990s, hundreds of recombinant proteins, many of them with pharmacological or therapeutical activities, have been expressed in transgenic plants. Old field trials included, for example, the production of: ***i)*** cholera toxin in alfalfa (Noble Foundation, 1992); ***ii)*** serum albumin, α- and β-globin, and procollagen in maize (Limagrain, 1998); ***iii)*** lactoferrin, antitrypsin, lysozyme, antithrombin and serum albumin, in barley (Washigon State University, 2001); ***iv)*** aprotinin and trypsinogen from cow, glycoprotein gp 120 from HIV, and enterotoxin subunit B from *E. coli*, in maize (ProdiGene, 2002).

More recently, SemBioSys Genetics Inc., a Canadian biotech company, developed a production platform in safflower (*Carthamus tinctorius L*) seeds: the recombinant proteins were fused to oleosins and stored in oil bodies, which allowed very simple purification methods. Two proteins produced in this system, Apo AI (the lipoprotein associated with HDL, 'good cholesterol') for prevention and treatment of cardiovascular disease, and human insulin, were undergoing clinical trials when the company went bankrupt in 2012, before they could be marketed.

Another example of a 'pharma crop' is Ventria Bioscience's transgenic rice, expressing two recombinant human milk proteins (lactoferrin and lysozyme) with anti-diarrhoea effects. The rice grains were processed into a powder to make oral rehydration solutions, which were planned to be the first commercialised transgenic over-the-counter 'medical food'. This product, however, never reached the market and at present several variants are in clinical trials for hospital treatment of more serious conditions, such as diarrhoea associated to antibiotic treatments, chemotherapy-induced diarrhoea, HIV-associated chronic inflammation or inflammatory bowel disease.

PRESENT SITUATION OF PLANT-BASED BIOPHARMACEUTICALS

For over 20 years, a large number of recombinant proteins have been produced in transgenic plants, many with potential clinical applications, proving the economic and technical advantages of 'molecular farming', over conventional production platforms using mammalian cell cultures. When referring specifically to plant-made biopharmaceuticals, however, in most cases this work has been limited to academic or 'proof-of-concept' stu-dies; until recently, no plant-produced pharmacological protein has been approved to be used in humans. Therefore, the recombinant proteins which are currently produced in plants are marketed as reagents for research or used in various industries: cosmetics, detergents, food, etc., but production of pharmaceutical proteins in GM plants is lagging far behind the fermenter-based systems of cultured mam-malian cells.

This is due, in part, to the long and expensive procedures (including clinical trials) required to bring to the market a new pharmaceutical protein for use in humans. Yet, regulatory and technical issues are in general more relevant than purely scientific advances. There are no clear specific rules applicable to the production of pharmaceuticals in plants, and it is very difficult to adapt those existing at present, established for such different biological systems as cells in *in vitro* cultures (similar regulatory problems exist in the case of the production of therapeutic proteins in transgenic animals). In addition, worldwide, there are only a few facilities authorised for the production of

recombinant proteins in plants according to 'good manufacturing practice' (GMP) for clinical development.

Nevertheless, in recent years, there has been a substantial boost in commercial development and potential applications of molecular pharming, and several specific products are currently undergoing clinical trials, at different phases (Stöger et al., 2015). Some of the specific milestones that mark this development are:

i) The approval by the FDA, in May 2012, of recombinant glucocerebrosidase (commercial name: 'Elelyso'), enzyme used to treat Gaucher's disease, a lysosomal storage disorder. The protein is produced by an Israeli company, Protalix Biotherapeuticals, in a carrot cell suspension culture (Tekoah et al., 2015). This was the first pharmaceutical recombinant protein produced in a GM plant system approved for human use... although the plant cell culture was not so different technically from an animal cell culture.

ii) Production of the experimental drug ZMapp by transient expression in leaves of *Nicotiana benthamiana*. ZMapp contains a combination of three humanised monoclonal antibodies that recognise a surface glycoprotein of the Ebola virus, and was proven to be effective against the virus in infected primates (Qiu et al., 2014).

iii) Production in stably transformed GM tobacco of mAb 2G12, a neutralizing anti-HIV monoclonal antibody. The antibody is used as a topical prophylactic to prevent virus infection (by vaginal application prior to sexual intercourse). The relevant authorities approved a phase I clinical trial, which demonstrated the safety of its use (Ma et al., 2015). This project, 'Pharma-Planta', funded by the EU Sixth Framework Programme, was used to establish and develop an approved manufacturing process for a recombinant plant-made pharmaceutical protein, according to 'good manufacturing practice'. The complete procedure, from gene to harvest, included the design of expression constructs, plant transformation, the generation of production lines, master and working seed banks and the detailed investigation of cultivation and harvesting parameters and their impact on biomass, product yield and intra/interbatch variability (Sack et al., 2015).

PERSPECTIVES

'Molecular pharming' will probably never replace the systems of animal cell cultures, much more developed, and where the industry has made major investments. Yet the advantages of using transgenic plants as biofactories for the production of biopharmaceuticals provide a very interesting market niche for specific products. GM plants may be the best choice to produce recombinant proteins with particular properties or for specific uses, which cannot be synthesised by the current platforms due to low efficiency or very high costs. Some of these specific applications could be:

i) the synthesis of products of higher pharmacological activity or better quality, for example by engineering in the GM plants specific glycosylation patterns

ii) the production of oral vaccines, encapsulated in a plant matrix

iii) the production of proteins that must be rapidly obtained, in response to an emergency – such as vaccines for pandemic diseases or a bioterrorist attack – using transient expression systems

iv) development of simplified purification steps, to reduce the cost of downstream processing

v) synthesis of proteins required in massive amounts... (e.g., human serum albumin, insulin, topical microbicides)

vi) ... or at a very low scale (e.g. for 'personalised medicine')

vii) for products that cannot be produced in cell cultures, for example toxic proteins

CONCLUSIONS

The use of plant biofactories for the commercial production of biopharmaceuticals cannot compete, in general, with the well-established, fermenter-based mammalian cell culture systems. Yet plant-based platforms represent an attractive alternative for the production of specific proteins that cannot be synthesised in mammalian cells, or when the particular characteristics of the desired product make the use of cell culture unsuitable or unprofitable. The scientific basis for production of recombinant proteins in transgenic plants are well established and, as described in the

previous paragraphs, 'molecular pharming' has several economic and technical advantages over cell culture systems. Further development of the technology is to be expected in the near future, once GMP ('good manufacturing practice') procedures for biopharmaceuticals production are more widely established for plant systems and approved by the competent authorities, so that the present regulatory limitations are overcome.

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