Session 7C

Biopharmaceuticals

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Production of recombinant biopharmaceuticals in plants – a potential solution for global health

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Plant-derived pharmaceuticals are poised to become a major development in biotechnology. The advantages they offer in terms of production scale and economy, product safety and ease of storage and distribution cannot be matched by any current commercial system. More importantly, they also provide the most promising opportunity to address Global Health care, with a focus on the poor in the developing world.

The first plant-derived pharmaceutical proteins (PDPs) have recently been licensed both in the USA and Europe and commercial release of these and the pipeline of products in final clinical trials will follow shortly. The roll-out of the technology would be greatly accelerated by the participation of major players from the pharmaceutical industry, however industry has been slow to become involved, for understandable reasons. Above all, it is widely recognized that those with most to gain from this technology are likely to be the poor in developing countries, where infectious diseases are common and modern medicines are scarce. Applications in the developed world that fully exploit the advantages of plants and form persuasive enough cases to make the pharmaceutical industry change to a completely new platform technology, are relatively scarce. Perhaps for the first time, we are confronted by the need to develop a commercial technology for humanitarian purposes, rather than commercial gain.

The production of pharmaceuticals, including vaccines in plants can potentially address many of the challenges posed by existing methods of production. The combination of low capital investment, low cost of goods, coupled with highly scalable manufacturing capability is particularly important for many products and will enable the development of new applications (such as passive immuno therapy with monoclonal antibodies) that are currently not achievable with fermenter based technologies.

The principal advantages of plant-based production systems are:

1. Scalability – No other production system offers the potential scalability of plants. Whilst some high-value products could be produced in sufficient amounts in plant cell culture in the future, contained technologies in greenhouses or growth at agricultural levels will allow product manufacture on a massive scale. This in turn will enable the design of new products and approaches in many areas, and in the medical arena, will offer the prospect of providing medicines and vaccines at a scale that could finally match the global health need.

2. Cost – Plants are cost-effective and easy to grow. So, the cost of raw goods will certainly be low. However, as this typically represents only a small percentage of the total cost of a product, this is not necessarily where the major financial savings are to be found.

The major cost attraction of plants is that the initial investment into a production line is significantly lower for plants, as compared with conventional fermenter facilities. Many observers have also noted that for a PDP the requirement for a major capital investment can be delayed until much later in the product development line.

Taken together, these cost advantages are exactly the requirement to allow the involvement of new organisations in biopharmaceutical production, particularly those from developing countries themselves.

3. Adaptability – Plant cells are higher eukaryotes, and therefore possess, like mammalian cells, an endomembrane system that allows them to produce extremely complex proteins such as monoclonal antibodies that are currently not feasible in, for example, microbial systems. Indeed, all the generally recognised forms of antibody and related engineered molecules have been successfully expressed in plants. In addition, there are examples of proteins that, at present, can only be produced in plants (for example secretory IgA antibodies, and recombinant immune complexes). Thus plants appear to be highly amenable to the production of a wide range of proteins.

4. Speed – The latest advances in plant biotechnology now allow large scale amounts of high quality recombinant proteins to be produced extremely rapidly. This has allowed at least three plant-based commercial ventures to develop technologies that will allow them to compete, for example, with existing systems for the production of influenza vaccine, or for the requirement for rapid scalability of products to respond to bio-terrorist threats.

Of course, not all pharmaceuticals will be appropriate, or need to be made in plant systems.

Those application areas that are likely to benefit most are:

1. Medicines that are required in very large quantities e.g. monoclonal antibodies, some sub-unit vaccines, HIV protein microbicides.

2. Medicines that can only be made in plants e.g. secretory IgA antibodies - at present this major class of antibody that is important for mucosal prevention of disease cannot be made efficiently by any other means.

3. Medicines that are specifically designed for production in plants e.g. recombinant immune complexes - with a growing understanding of the mechanisms of protein production in plants, has come the ability to engineer molecules with enhanced immunological properties.

Challenges and opportunities of plant derived biopharmaceuticals

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Introduction

Many of today's pharmaceuticals are derived from plants, but protein drugs are exceptional in that they are predominantly of mammalian origin. They are usually produced in cultured human or rodent cells, which provide almost the correct biochemical environment. The biopharmaceutical industry has therefore evolved to view mammalian cells as the gold standard for production. The focus on mammalian cells has had a negative impact on the use of plants, despite their many potential advantages including the prospect of inexpensive, large-scale biopharmaceutical production without sacrificing product quality or safety.

The first plant-derived pharmaceutical products have now been approved but these represent a tiny proportion of the products in development, products which could have a profound impact on the cost and availability of medicines to those most in need. The obstacles currently preventing the rapid commercialization of plant-based expression platforms are technical and political. The technical hurdles are falling the most quickly, since these revolve around two major issues – quality and quantity.

Quality

Recombinant proteins produced in plant cells are identical at the polypeptide level to those produced in mammals. The ability of plants to fold and assemble complex human proteins has been demonstrated by the successful production of functional antibodies and other multimeric proteins. However, although the protein synthesis and folding pathways are highly conserved between plants and animals, there are some differences in the capacity for post-translational modification, especially in glycan structure: plant-derived recombinant human glycoproteins tend to contain the carbohydrate groups $\beta(1\rightarrow2)$ xylose and $\alpha(1\rightarrow3)$ fucose, which are absent in mammals, but generally lack the terminal galactose and sialic acid residues that are found on many native human glycoproteins. Since glycan structures can impact on the solubility, stability, immunogenicity and biological activity of recombinant proteins, the 'humanization' of glycan structures produced in plants has been attempted by protein targeting and strain development to prevent the addition of plant glycans.

Quantity

The quantity of recombinant protein produced in plants is improving all the time, through the development of strategies to increase yields and protein stability. Although there are spectacular successes, many proteins expressed in plants accumulate at low levels, i.e. less than 1% of total soluble protein (TSP). This seems to be a problem largely limited to nuclear transgenic plants, since very high levels have been achieved using the chloroplast expression system and viruses, the latter achieving as much as 80% TSP or 5 mg per gram fresh weight of plant tissue. For nuclear transgenics, the most important component of the expression construct is the promoter used to control the transcription of the transgene. With a strong promoter, the yield of recombinant protein also depends on its subcellular

compartment in which a recombinant protein accumulates influences its folding, assembly and post -translational modification. Product yields can be increased not only through construct design, but also through the implementation of a selection and backcrossing program once transgenic plants are available. In the case of maize, it has been possible to increase the yield of recombinant avidin over 150-fold in eight generations.

Purity
For the production of clinical grade recombinant proteins, downstream processing steps need to meet the standards that have been set for other biopharmaceutical production systems, including a strict regime of quality assurance and quality control to achieve approval of regulatory agencies. The initial stages of processing display the greatest variability and have to be optimized in a system-specific manner. Disruption of cell walls and membranes is the first post-harvesting step, but different tissue types (leaves, seeds, fruits etc.) require different forms of treatment. After cell disruption, clarification of the extract is often carried out by dead-end or cross-flow filtration, some-times preceded by bulk cell mass removal using a decanter, plate separator or centrifuge. Once a clarified feed is available, the downstream steps become more product orientated, although it may be necessary to introduce purification steps that cater for specific impurities found in plants such as phenolics and proteases.

The future
The production of recombinant pharmaceuticals in plants is advantageous, theoretically offering unlimited production scales at unprecedented low manufacturing costs. We are beginning to overcome the technical limitations such as low yields, instability and non-authentic glycan structures that place hurdles along the path towards commercialization, but more needs to be done to convince industry that plants represent a true alternative to CHO cells and bacteria. Despite the further limitations of a formative and, in some cases, restrictive regulatory framework, the potential of plant-derived pharmaceutical proteins can be seen in the rich IP landscape and the multiple cross-licensing and collaborative ventures that are possible between companies developing production platforms, extraction and separation technologies and those with experience in the latter stages of drug development and marketing. The welcome announcement of the first approved plant-derived veterinary vaccine may open the way for plant-derived therapeutic and diagnostic proteins to become established as a competitive and valuable source of novel medicines.

Biopharming in plants: science, regulation and commercialisation

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Bio-pharming of pharmaceutical and industrial compounds in plants offers an attractive alternative to mammalian based pharmaceutical and vaccine production. Several plant-based products are already on the market (Prodigene's avidin, β-glucuronidase, trypsin generated in GM maize; Ventria's lactoferrin generated in GM rice). Numerous products are in clinical trials (collagen, antibodies against tooth decay and non-Hodgkin's lymphoma from tobacco; human gastric lipase, therapeutic enzymes, dietary supplements from maize; Hepatitis B and Norwalk virus vaccines from potato; rabies vaccines from spinach; insulin from safflower, dietary supplements from *Arabidopsis*). The initial production platforms for plant-based pharmaceuticals were selected from conventional crops, largely because an established knowledge base already existed. Tobacco and other leafy crops such as alfalfa, lettuce and spinach are widely used as leaves can be harvested and no flowering is required. Many of these crops can be grown in contained greenhouses. Potato is also widely used and can also be grown in contained conditions.

Plant based pharmaceuticals present a particular set of problems for the regulators. The contamination of crops destined for consumption by humans or livestock by genes that express potentially harmful compounds is a cause for real concern, as has already been demonstrated by the 'Starlink' episode, where material from a non-approved line of *Bt* corn was found in processed food products in the US. This was followed in 2002 by identification of corn expressing trypsin as 'volunteers' in the following year's soybean crop. A number of novel approaches are being investigated and adopted in order to reduce this risk. These relative merits of these various methods will be discussed.

Physical containment in greenhouses and growth rooms is suitable for some crops (tomatoes, lettuce) and for research purposes. There are obvious limitations of the scale of physical containment strategies, addressed in part by the development of large underground facilities in the US and Canada. The additional resources required to grow plants underground incurs high costs that in the long run may negate any advantage of GM for commercial production.

In addition, various biological methods are available to reduce or eliminate gene transfer via pollen or any other route, with the choice of species being the most obvious consideration.

Natural genetic containment has been adopted by some companies through the selection of non-food/feed crops as biopharming platforms (algae, moss, duckweed) or organisms with no wild relatives present in the local flora (safflower in the Americas). The expression of pharmaceutical products in leafy crops enables growth and harvesting prior to and in the absence of flowering (tobacco, alfalfa, lettuce, spinach).

Aquatic bioreactors of some non-crop species (algae, moss, and duckweed) expressing pharmaceutical products have been adopted by some biotechnology companies.

Plant cells or plant parts may be transformed and maintained in culture to produce recombinant products in a contained environment. Plant cells in suspension or *in vitro*, roots, root cells and guttation fluid from leaves may be engineered to secrete proteins that may be harvested in a continuous, non-destructive manner. Strategies such as these remain developmental and have not been commercially adopted at present.

Transient expression produces GM products from non-GM plants using trait induction via the utilisation of bacterial or viral vectors. These vectors introduce the trait into specific tissues of whole plants or plant parts, but do not insert them into the heritable genome. There are some limitations of scale and the field release of such crops will require the regulation of the vector.

Transgenically controlled containment strategies range in their approach and degree of development. Plastid transformation is relatively well developed but is not suited to all traits or crops and does not offer complete containment. Male sterility is well developed across a range of plants but has limitations in its application for fruit/seed bearing crops. It has been adopted in some commercial lines of oilseed rape despite not preventing escape via seed. Conditional lethality can be used to prevent flowering or seed development following the application of a chemical inducer, but requires 100% induction of the trait and sufficient application of the inducer to all plants. Equally, inducible expression of the GM trait requires equally stringent application conditions. Such a method will contain the trait but will allow the escape of a non-functioning transgene. Seed lethality (terminator technology) is the only strategy at present that prevents transgene movement via pollen and seed, but due to public opinion against the concept it has never been trialled in the field and is no longer under commercial development.

Methods to control flowering and fruit development such as apomixis and cleistogamy will prevent crop-to-wild and wild-to-crop pollination, but in nature both of these strategies are complex and leaky. None of the genes controlling these traits have as yet been identified or characterised and therefore have not been transgenically introduced into crop species. Neither of these strategies will prevent transgene escape via seed and any feral apomicts that form are arguably more likely to become invasives.

Transgene mitigation reduces the fitness of initial hybrids and so prevents stable introgression of transgenes into wild populations. However, it but does not prevent initial formation of hybrids or spread to non-GM crops. Such strategies could be detrimental to wild populations and have not yet been demonstrated in the field. Similarly, auxotrophy prevents persistence of escapes and hybrids containing the transgene in an uncontrolled environment, but does not prevent transgene movement from the crop.

Recoverable block of function, intein *trans*-splicing and transgene excision all use recombinases to modify the transgene *in planta* either to induce expression or to prevent it. All require optimal conditions and 100% accuracy to function and none have been tested under field conditions as yet. All will contain the GM trait but all will allow some non-native DNA to escape to wild populations or to non-GM crops.

In the final analysis the choice of any specific combination of crop and production method will be determined, not by scientific opportunity but rather by commercial considerations of rates of return on investment.

Generating novel phytochemicals through biocatalysis

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Plant-derived natural products often have desirable pharmaceutical properties and historically have proved to be a fertile source of novel pharmacophores. Chemical modifications of these natural products is then often employed to enhance their uptake, efficacy at the site of action and stability. Relatively simple modifications can be achieved through synthetic chemistry. In other cases regio- and stereo-selective chemical modification of the natural product is problematic and not commercially viable. As such enzyme-mediated structural modification can represent a useful alternative approach to chemical synthesis in improving the medicinal activity of natural pharmaceuticals.

Acylation can radically affect the bioactivity of secondary metabolites and is often seen in synthetic second generation derivatives of natural products. In particular, fluorinated acyl moieties have been found to impart desirable pharmaceutical characteristics upon natural compounds. For example Flecainide, an antiarrhythmic agent and 2-(fluorobenzoyl)-Paclitaxel an anticancer agent, have modified and desirable drug activities as compared with the parent natural products. As the most electronegative atom in the periodic table, fluorine simultaneously modulates electronic, lipophillic and steric parameters of compounds, all of which can critically influence their bioactivity. We have therefore investigated the use of enzymes to selectively introduce fluorine–containing acyl groups into natural product acceptors to diversify their chemical range and efficacy.

The strategy adopted has been to biosynthetically activate synthetic fluorinated acyl groups by enzymically forming the respective fluoroacyl–CoA esters and then use these donors as coupled substrates for acyltransferases which acylate the selected natural product acceptor in a regio-specific manner. This modification pathway consists of a CoA ligase co-expressed with the acyltransferase, such that by feeding in acyl donors and acceptors, acylated products are biosynthetically formed in a 'single pot' reaction. To test this system, a coumaroyl-CoA ligase and BAHD aromatic acyltransferase active toward antioxidant anthocyanin and flavonol substrates were cloned from *Arabidopsis thaliana* and *Gentiana triflora* respectively. The CoA ligase was found to accept a range of fluorinated phenylpropanoid precursors and so form the respective CoA thioesters. While the conversion of the non-natural substrates were lower than that determined with the natural substrates, overall, the synthetic acyl donors were selectively inserted into the structure of anthocyanin acceptors via the activity of a BAHD acyltransferase at a comparable rate to the non-fluorinated hydroxylated acylating moieties (Table 1). Thus, one–pot biosynthesis was found to be important in preventing the enzymatic hydrolysis of the labile coenzyme A thioesters and considerably improved the overall yield of the biotransformation.

The search for novel biocatalysts, donors and acceptors and the combined use of acyl donor-specific CoA ligases and acyl – acceptor specific acyltransferases to create novel bioactive plant products will be discussed.

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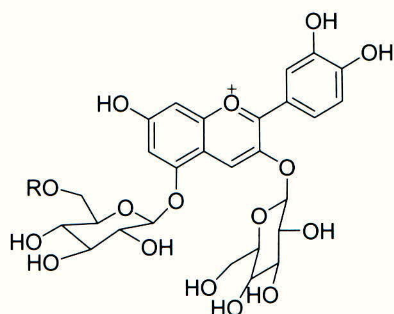
Table 1 Biosynthesis of anthocyanin natural products bearing non – natural acylating moieties



Acyl acceptor

R group	*Specific activity (nkat mg^{-1} protein) Acyltransfer*
	42.4 +/- 1.6
	48.9 +/- 0.9
	0
	38.0 +/- 1.0
	40.8 +/- 1.4
	0

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The production of very long chain fatty acids in transgenic plants

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There is now much evidence as to the importance of very long chain polyunsaturated fatty acids (VLC-PUFAs) in optimal human nutrition. In particular, the omega-3 C_{22} docosahexaenoic acid (DHA) plays a key role (in conjunction with the omega-3 C_{20} VLC-PUFA eicosapentaenoic acid, EPA) in reducing the risk of cardiovascular disease and metabolic syndrome. Similarly, DHA and the omega-6 C_{20} VLC-PUFA arachidonic acid (ARA) are known to play a key role in aspects of neonatal development, in particular the development of the retina and brain. It is on account of these observations that ARA and DHA are now routine components of infant formula milks; this also represents a high-value market for these fatty acids.

Current sources of fatty acids such as ARA and DHA are derived either from free-living sources or via culture. In the case of DHA (and EPA), the primary source is marine fish oils, whereas for ARA this is fermentative culture of the filamentous fungus *Mortierella alpina*. For fish oils there are a number of pressing concerns regarding the sustainability of these natural resources, since there is considerable evidence as to the global decline in wild fish stocks (mainly due to human over-exploitation). Equally, there is now evidence that the marine environment contains a number of pollutants (such as heavy metals, PCBs, etc) which accumulate in the aquatic food webs and can be detected in fish oils. Such concerns have even led to some health-protection agencies recommending that pregnant women do not consume fish, in spite of the benefits of fish oils. In the case of ARA production, continuous culture of *M. alpina* requires dedicated facilities which have an associated environmental footprint and also have constraints on up-scaling.

In view of all of the above, we believe that there is a clear need for an alternative source of VLC-PUFAs for human health and nutrition, preferably one which can demonstrate improved sustainability and a reduced carbon footprint. We have therefore investigated the feasibility of using transgenic plants as 'bioreactors' or green factories for the synthesis and accumulation of VLC-PUFAs such as ARA, EPA and DHA. Such an approach has a number of potential advantages over current sources, being sustainable, free from environmental pollution and also contributing to the capture of greenhouse gases. However, not only does the (reverse) engineering of plants with the capacity to synthesize VLC-PUFAs represent a daunting technical challenge, there are also a number of regulatory and consumer issues to be considered in the use of GM approaches to produce these important fatty acids.

In terms of generating transgenic plants with the capacity to synthesize C_{20+} VLC-PUFAs such as ARA and EPA, it needs to be highlighted that there is no known species of higher plant which accumulates these fatty acids. Oilseed crops accumulate high levels of the C_{18} precurors such as the omega-6 linoleic acid (LA) and the omega-3 α-linolenic acid (ALA). These two fatty acids serve as substrates for the transgene-derived VLC-PUFA enzyme activites which convert the C_{18} fatty acids into C_{20} and C_{22} forms by sequential desaturation and elongation.

Considerable progress has now been made in the identification and functional characterization of the VLC-PUFA desaturases and elongases from a wide range of organisms, predominantly marine algae which represent the primary producers of these fatty acids and the base of the aquatic VLC-PUFA food webs. These gene sequences provide a toolkit with which to attempt their co-ordinate expression in transgenic plants, with the goal of directing the synthesis of VLC-PUFAs which will be accumulated in the seed oil triacylglycerols. Thus, the challenge is two-fold: firstly, to obtain efficient heterologous synthesis of these non-native fatty acids, and secondly to ensure that these products are efficiently removed from their site of synthesis and compartmentalized in storage oil reserves.

The minimum number of transgenes required to direct the conversion of endogenous plant C_{18} fatty acids to a C_{20} VLC-PUFA is three, namely the Δ6-desaturase, Δ6-elongase and Δ5-desaturase. Several independent studies have confirmed that expression of this minimal gene set will result in the accumulation of ARA and EPA, though the levels of these non-native fatty acids appears to be influenced by a number of different factors. Firstly, the endogenous levels of LA and ALA will play a role in determining the levels of ARA and EPA, respectively. Secondly, it is now clear that a major bottleneck in the pathway is the so-called 'substrate dichotomy' which is inherent in VLC-PUFA biosynthesis. Thus, fatty acid desaturation utilizes phospholipid-linked substrates whereas elongation requires acyl-CoA substrates. It is very likely that the organisms from which the genes for VLC-PUFA biosynthesis were obtained contain additional (secondary) activities which facilitate the required exchange between these two metabolic pools to ensure efficient synthesis. However, in transgenic plants engineered with only the primary biosynthetic genes, the ability to bypass this bottleneck is dependent on endogenous acyl-exchange enzymes which are involved in other aspects of lipid metabolism but have some capacity to recognize these non-native fatty acids as substrates. It is likely that the considerable observed variation between different transgenic plant species to accumulate ARA and EPA is mediated through differences in their endogenous acyltransferases. A final factor is that it is also likely to play a role is that of 'negative' activities present in plants which act to channel VLC-PUFA intermediates away from the biosynthetic pathway and therefore reducing overall efficiency.

Using all of the above information, we have embarked on the rational metabolic engineering of transgenic plants with the capacity to accumulate high value VLC-PUFAs. Such an approach is likely to be iterative in nature, since we consider it likely that more than one factor will determine the efficiency of this heterologous pathway in any given transgenic oilseed species. We believe that by taking this approach it will be possible to regenerate transgenic plants which accumulate 15-25% VLC-PUFAs in their seed oil. This will represent a valuable commodity with which human health can be improved whilst clearly demonstrating enhanced sustainability compared with current sources. Whilst it remains to be determined if such a GM product might be acceptable to the European consumer, it is to be hoped that transgenic plants containing such traits might help in demonstrating the value of this technology.