

Transgenic plants as bioreactors

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Recent advances in molecular and cellular biology have led to the development of technology to engineer plants that are capable of producing a wide variety of products. These include products for pharmaceutical applications, like vaccine antigens, antibodies, antibody-derived fragments and other therapeutic proteins. It is possible to alter endogenous metabolic pathways to obtain new products, like plastics, or to confer novel traits, such as improved nutritional value.

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Introduction

Plant biotechnology, based on the recombinant DNA technology, is paving the way to produce novel biological molecules in crop plants. Transgenic plants have significant potential in the bioproduction of complex human therapeutic agents due to ease of genetic manipulation, lack of potential contamination with human pathogens, conservation of eukaryotic cell machinery mediating protein modification and low cost of biomass production. In recent years, several proteins have been successfully produced in plants, which include human serum albumin, haemoglobin, monoclonal antibodies, viral or bacterial antigens (vaccine), enkephalin and trichosanthin^{1,2}.

Two basic strategies have been used to produce recombinant proteins in plants: (i) generation of the transgenic plants by stable integration of a transgene in the plant genome, and (ii) transient expression of the transgene by using plant viruses as vectors. An excellent and widely used technique for the production of transgenic plants is the *Agrobacterium*-mediated transformation, where the genes of interest are first introduced into *Agrobacterium tumefaciens* and, subsequently, plant cells are infected to transfer the gene of interest to the plant genome. The other techniques used for direct gene transfer are electroporation, polyethylene glycol-mediated gene uptake and particle bombardment. In a transgenic plant, it is necessary to achieve the highest possible level of foreign protein production. This requires the use of a strong promoter sequence, which enhances

the expression of the product of interest. However, there are some disadvantages also, offered by plants used as bioreactors, along with various advantages.

Advantages

1. It is simpler, cost-effective and faster to produce transgenic plants as compared to transgenic animals.
2. Plant pathogens do not infect humans or animals.
3. Plants usually mature after one season of growth and it is possible to bring out the product to the market within a short time.
4. Plants that generate large biomass like corn and tobacco can produce large amounts of genetically engineered products.
5. Proteins can be indefinitely stored in seeds with little reduction in biological activity.

Disadvantages

1. Differences in codon usage between plants and prokaryotes can lead to inefficient expression of prokaryotic proteins in plants.
2. Plants may attach different polysaccharides to proteins.
3. Some plants produce allergenic compounds.

Production of Vaccine Antigens in Plants

The aim of vaccination is to prevent infectious diseases. It can be considered as one of the most successful breakthrough of this century in the medical field. The principle of vaccination involves mimicking an infection in such a way that the specific natural defence mechanism of the host against the pathogen gets activated but the host remains free of the disease that normally results from such infection. Vaccination is also referred to as 'active immunization', since host immune system is

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'activated' to respond to infection through humoral and cellular responses, resulting in acquired immunity against the particular pathogen. Further, most of the human pathogens that cause respiratory and intestinal diseases initiate infection at mucosal surfaces, where they encounter the mucosal immune system (MIS). Mucosal response is achieved more efficiently by oral instead of parenteral delivery of the antigens. Immunity *via* mucosal tissues can be induced by delivering the antigens with live, replicating agents, such as attenuated or mutant strains of recombinant bacteria or viruses. Many of these attempts have had limited success and the use of live vaccines raises obvious safety issues. Most soluble, non-viable antigens are subject to degradation in the gastrointestinal tract due to acidic and proteolytic environment of the gut. Thus, expressing the antigen in plant cell can be considered as a means of bioencapsulating protein for oral delivery. Plant cell wall consisting of cellulose, proteins and pectin will serve as barrier to enzymatic degradation, and cell membrane and internal organelle membranes provide further protection.

The launch of vaccines for children, initiative by World Health Organisation along with few other organisations in 1990, had set the goal for production of vaccines that are safe, heat stable, easy to administer and widely accessible. The expression of vaccine antigen gene in edible plant tissues meets all of these requirements, at least for diseases involving infection at mucosal surfaces. The first report on the use of plants for the production of edible vaccine appeared in the form of a patent application by Curtiss and Cardineau³. They expressed the *Streptococcus mutans* surface protein antigen A (SpaA) in tobacco. Since then various antigenic determinants against viral and bacterial pathogens have been expressed in transgenic plants (Table 1). Few instances in which detailed investigations have been going on are given below.

***Escherichia coli* Labile Toxin**

Escherichia coli labile toxin (LT), which is responsible for causing diarrhoea, is composed of a 27 kDa A subunit and five 11.6 kDa B subunits that pentamerise. The B pentamer (LT-B) has been used as a vaccine component, as antibodies against this would block toxin activity. LT-B was expressed in transgenic potato¹⁰. However, the maximum expression level achieved represented 0.01% of total soluble protein. Mice fed with such transgenic tuber

developed an oral immune response. Further, a synthetic gene was created for LT-B that contained plant-preferred codons¹¹. As a result, the expression level increased to 0.19% of the total soluble protein. In a clinical trial, transgenic tubers expressing LT-B, when fed to human volunteers, showed the development of serum and mucosal immune response to LT-B¹². In another study, the recombinant LT-B, produced in potato tubers, was found immunogenic and, on oral administration, elicited a systemic and local IgA response in parenterally primed but not in naïve animals¹³. A synthetic gene encoding, a variant of LT-B, was also expressed in transgenic corn and oral administration of transgenic corn elicited serum and mucosal immune responses in mice¹⁴.

***Vibrio cholerae* Toxin**

Cholera toxin (CT) is very similar to *E. coli* LT. The genes encoding CTA and CTB were amplified by PCR and then cloned into plant expression vectors¹⁵. Expression of these genes was controlled by the CaMV 35S promoter and a reiterated 35S enhancer. Variants of the *ctxA* gene were prepared and consisted of mature *ctxA* coding sequence preceded by 1) the native *ctxA* signal peptide sequence, or 2) coding sequence for a signal peptide of a murine origin. The third construct included a single amino acid exchange in order to eliminate enzymatic activity of *ctxA*. Transgenic tobacco plants, containing *ctxA* preceded by native/murine signal peptide, expressed CTA. The protein encoded by mutated *ctxA* was expressed at lower levels and was of higher molecular weight (29 kDa), which could probably represent uncleaved protein. Further, transgenic plants containing *ctxB* genes expressed CTB protein in leaves. However, only monomeric CTB could be detected. In potato tubers, CTB was expressed at the level of 0.3% of total soluble protein¹⁶. The transgenic potato tubers when fed to mice, serum and intestinal CTB specific antibodies were induced¹⁷. CTB was also expressed in tobacco chloroplasts at a level ranging between 3.5 and 4.1% of the total soluble protein¹⁸. Keeping in view the concept of edible and heat-labile nature of vaccine, CTB was expressed in tomato in authors' laboratory¹⁹. The antigen was found to organize in pentameric form capable of interacting with G_{M1} ganglioside as is known to happen in natural condition. The transgenic tomato fruits can be eaten fresh and can be available in all seasons. Thus, such material could possibly be evaluated for inducing immunity by oral delivery.

Table 1—Stable expression of certain antigenic determinants in transgenic plants

Disease	Causitive agent	Antigen	Plant	Level of expression	Immunization	Reference
Hepatitis	Hepatitis B virus	Hepatitis B virus surface antigen (HBsAg)	Tobacco	Up to 0.01% TSP	—	Mason <i>et al</i> ⁴
			Lupin callus and lettuce leaves	Up to 150 ng/g fresh weight in lupin callus and up to 5.5 ng/g fresh weight in lettuce leaf	Mice immunized intraperitoneally developed anti HBsAg response	Thanavala <i>et al</i> ⁵
			Potato	1.1 µg/g tuber	Mice fed lupin callus developed HBsAg specific antibodies. Human volunteers fed transgenic lettuce, developed specific serum IgG	Kapusta <i>et al</i> ⁶
			Potato	8.35 µg/g fresh tuber	Mice fed transgenic tubers developed HBsAg specific antibody which was boosted by intraperitoneal delivery of a subimmunogenic dose of commercial HBsAg vaccine	Richter <i>et al</i> ⁷
					Orally immunized mice developed antibody response above the minimum protective level. Parenteral boosting generated a long lasting secondary antibody response	Kong <i>et al</i> ⁸
Rabies	Rabies virus	Rabies virus glycoprotein	Tomato	1-10 ng/ mg TSP	—	McGarvey <i>et al</i> ⁹
Traveller's diarrhoea	<i>Escherichia coli</i>	Labile toxin B subunit (LT-B)	Potato	0.01% TSP	Oral immunization elicited immune response in mice	Haq <i>et al</i> ¹⁰
			Potato	0.19% TSP	Mice fed transgenic tubers developed anti-LTB antibodies and were partially protected against challenge with orally delivered labile toxin	Mason <i>et al</i> ¹¹
		Potato	17 µg rec LT-B per gram tuber	Subcutaneous immunization elicited high anti-LT titres in mice. Oral immunization with a total dose of 780 µg of recLTB per mouse did not evoke detectable IgG1 or IgA antibody titres in serum. However, oral boosting of primed animals induced an immune response	Tacket <i>et al</i> ¹²	
		Synthetic labile toxin B subunit	Potato	17 µg rec LT-B per gram tuber	Human volunteers orally immunized with transgenic tubers developed anti-LT IgG and sIgA	Lauterslager <i>et al</i> ¹³

		Synthetic labile toxin B subunit	Corn	-		Orally immunized mice developed anti-LTB specific serum IgG and IgA in fecal material	Streatfield <i>et al</i> ¹⁴
Cholera	<i>Vibrio cholerae</i>	Cholera toxin subunits A & B	Tobacco	—			Hein <i>et al</i> ¹⁵
		Cholera toxin B subunit (CTB)	Potato	0.3% TSP		-	Arakawa <i>et al</i> ¹⁶
						Mice fed transgenic tuber elicited serum and mucosal anti-CTB antibodies	Arakawa <i>et al</i> ¹⁷
		Cholera toxin B subunit	Tobacco chloroplasts	4.1% TSP		—	Daniell <i>et al</i> ¹⁸
		Cholera toxin B subunit	Tomato	0.02% TSP in leaves and 0.04% TSP in fruits		—	Jani <i>et al</i> ¹⁹
Gastroenteritis	Norwalk virus (NV)	Norwalk virus capsid protein	Tobacco, potato	0.23% TSP of leaf and tuber		Oral immunization led to development of serum IgG and secretory IgA specific for NV in mice	Mason <i>et al</i> ²⁰
Porcine diarrhoea	Transmissible gastroenteritis virus (TGEV)	Spike protein S of TGEV	<i>Arabidopsis thaliana</i>	0.06-0.03% TSP		Mice immunized intramuscularly developed specific antibody response and neutralized virus infection	Gomez <i>et al</i> ²¹
			Tobacco	0.1-0.2% leaf TSP		Swine were immunized intraperitoneally with tobacco leaf extracts. Strong TGEV specific immune response was elicited	Tuboly <i>et al</i> ²²
			Potato	0.02-0.07% TSP in tubers		Intraperitoneal and oral immunization in mice led to induction of specific serum antibody response	Gomez <i>et al</i> ²³
Foot and Mouth disease	Foot and Mouth disease virus (FMDV)	VP1 structural protein	<i>Arabidopsis thaliana</i>	-		Intraperitoneal injection of total protein extracted from 50-100 mg leaf resulted in specific antibody response to VP1 and intact FMDV	Carrillo <i>et al</i> ²⁴
		VP1 135-160 peptide fused to β GUS	Alfalfa	0.5 -1 mg/g TSP		Intraperitoneal immunization of mice induced strong anti-FMDV response to VP1 135-160 synthetic peptide, VP1 and to purified FMDV. Mice were completely protected against experimental challenge with virulent virus	Dus Santos <i>et al</i> ²⁵
Rabbit hemorrhagic disease	Rabbit hemorrhagic disease virus (RHDV)	VP60 structural protein	Potato	0.3% TSP in leaf and comparable level in tubers		Rabbits were immunized subcutaneously followed by two intramuscular boosters. High anti VP60 antibody titres were observed and rabbits were fully protected against hemorrhagic disease	Castanon <i>et al</i> ²⁶

Cytomegalovirus infection	Human cytomegalovirus (HCMV)	Glycoprotein B complex (gB)	Tobacco	Up to 658 ng gB/g dry seed material	-	Tackaberry <i>et al</i> ²⁷
Lower respiratory tract disease	Respiratory syncytial virus (RSV)	RSVF protein	Tomato	12.68 µg/g fresh fruit weight for fruit specific <i>E8</i> promoter and 9.01 µg/g fresh fruit weight for expression driven by CaMV 35 <i>S</i> promoter	Oral immunization of mice resulted in serum IgG and IgA and mucosal IgA induction	Sandhu <i>et al</i> ²⁸
Measles	Measles virus	Measles virus hemagglutinin H protein	Tobacco	—	Intraperitoneal and oral immunization of mice resulted in specific antibody response with neutralizing activity against the virus	Huang <i>et al</i> ²⁹
Enteric infections	Rotavirus	22 amino acid epitope of murine rotavirus was fused N terminally to CTB. <i>E. coli</i> fimbrial colonization factor CFA/I was fused N terminally to CTA2	Potato	3.3 µg recombinant fusion protein per g fresh transgenic tuber	Orally immunized mice generated serum and intestinal antibody against pathogen antigens. When mice were challenged with rotavirus, diarrhoea symptoms were reduced in severity	Yu and Langridge ³⁰
Parvoviral infection in dogs and minks	Canine parvovirus (CPV)	A 21-mer peptide from VP2 capsid protein fused to β GUS	<i>Arabidopsis thaliana</i>	0.15- 3.3% TSP	Mice immunized intraperitoneally or orally developed specific immune response for the peptide and CPV	Gil <i>et al</i> ³¹
Bovine pneumonia pasteurellosis or shipping fever	<i>Mannheimia haemolytica</i> A1	A short derivative of leukotoxin, Lkt 50, fused to modified green fluorescent protein	White clover	Up to 1% TSP	Intramuscular immunization of rabbits led to the production of antibodies that neutralized authentic leukotoxin	Lee <i>et al</i> ³²
Malaria	<i>Plasmodium falciparum</i>	C-terminal region of merozoite surface protein (PfMSP1 ₁₉)	Tobacco	—	—	Ghosh <i>et al</i> ³³

TSP = Total soluble protein

Hepatitis B Virus Surface Antigen

Hepatitis B virus infection causes acute or chronic hepatitis, and hepatocellular carcinoma. The first vaccine developed, consists of hepatitis B surface antigen (HBsAg). This antigen is produced in large amounts in liver cells of infected individuals. Tobacco plants were genetically transformed with the gene

encoding HBsAg⁴, which was driven by CaMV 35S promoter with double enhancer linked to tobacco etch virus (TEV) 5' non-translated leader sequence. HBsAg expression levels ranged up to 66 ng/mg of soluble protein. The immunogenicity of tobacco derived HBsAg was tested intraperitoneally in mice. HBsAg-specific antibodies of all IgG subclasses as

well as IgM antibodies were produced. The plant derived HBsAg could also prime T cells *in vivo* that could be recalled *in vitro* to proliferate upon stimulation with the immunogen. Lupin and lettuce were created for expressing HBsAg at levels of 150 ng/g fresh weight in lupin callus and 5.5 ng/g fresh weight in lettuce leaf⁶. Mice fed with transgenic callus elicited HBsAg specific IgG response. Human volunteers, fed with transgenic lettuce, developed specific serum IgG response to HBsAg. Transgenic potatoes were also developed expressing the hepatitis B surface antigen⁷. Mice fed with transgenic tuber developed a primary immune response, which was boosted by intraperitoneal delivery of a single subimmunogenic dose of commercial HBsAg.

Although expression of many candidate genes holds great promise (Table 1), their application and utility as products are yet to be established, as development of vaccines to advanced stage has not been reported in most of these cases. However, encouraging results have been obtained in challenge studies as also in clinical trials conducted with human volunteers.

Development of Tolerance to Edible Antigens

‘Oral tolerance’ is generally characterised by the fact that an animal, fed an antigen at a certain level, becomes refractory or has diminished capacity to develop an immune response, which it normally develops when exposed to the same antigen by a systemic route. This could have a negative consequence, as it would lead to increased likelihood of disease rather than prevention. However, the concept of ‘oral tolerance’ has been used to advantage in suppressing insulinitis in NOD (non-obese diabetic) mice³⁴. Insulin-dependent diabetes mellitus is an autoimmune disease in humans. This involves lymphocytic infiltration of Islets of Langerhans, which is followed by destruction of insulin producing β cells. Several antigens, including insulin have been predicted as potential targets. Transgenic potato plants, synthesizing human insulin and its CTB conjugate, suppressed diabetes in NOD mice. This orally administered form of insulin did not have any metabolic effect on blood glucose as it got degraded but it might facilitate orally induced tolerance by creating smaller protein fragments that were better able to interact with GALT (gut associated lymphoid tissue). T cells that adoptively transfer suppression of experimental autoimmune diabetes following oral administration of antigen were triggered in an

antigen-specific manner. These cells mediated their effect by release of the cytokine, transforming growth factor β (TGF β), an antigen non-specific suppressor, in close proximity to effector cells that in turn down regulated the local inflammatory processes in pancreas³⁵.

Antibody Production in Plants (Plantibody)

Hiatt *et al* were the first to demonstrate the production of antibodies in plants³⁶. Since then, a number of groups have expressed plantibodies, either to modify plant performance, such as pest resistance³⁷, or with a view to exploiting plants as bioreactors for the large scale production of antibodies³⁸ for the following reasons:

1. Plants can assemble heavy and light chains into complete antibodies.
2. Plants permit appropriate post-translational modification for the production of antibodies.
3. Several groups have expressed complete antibody by targeting the antibody *via* endoplasmic reticulum to apoplast, the extracellular aqueous region in which hydrolytic degradation is minimal and antibodies secreted into it can accumulate in a relatively stable environment. The extraction of antibody is also simpler and can be achieved by conditions milder than those required for proteins located elsewhere.
4. Genetically stable seed stocks of antibody-producing plants can be produced and stored indefinitely at low cost; the seed stock can be converted into a harvest of large quantity of antibody within one growing season.

Production of a fully functional antibody in plants is not a very straightforward task because of the multi-subunit structure of antibody molecule. Moreover, expression of a complete antibody may not be required for many applications. For example, production of antigen binding domain as present in single chain Fv or Fab molecules may be enough to block binding of a pathogen or a virulence factor secreted by the pathogen and thus may limit the spread of the infection. However, a monovalent antibody fragment may have reduced affinity for binding to antigens as compared to a bivalent F(ab)₂ fragment or a complete antibody. Further, divalent nature may be required for aggregation of cells or bacteria in some cases. Production of these fragments without complete constant region might be sufficient

for the conditions where attenuation of the function of the antigen is required. Different antibody fragments have been expressed in plants for blocking actions of phytochrome A³⁹, abscisic acid⁴⁰ as well as for sequestration of organic pollutants⁴¹. However, antigen-antibody binding alone is not enough in some cases and secondary effector functions, attributed to constant region, are required. These functions include activation of complement and binding to phagocytes. Other features, which reside in constant region, are sites for glycosylation, placental transfer, association with J chain and secretory component. Ma *et al*⁴² were the first to produce functional, high molecular weight secretory immunoglobulin in transgenic tobacco, which acts against adhesion protein of *Streptococcus mutans* known to cause tooth decay. They had cloned the heavy and light chains of murine antibody, murine joining chain and a rabbit secretory component into separate transgenic tobacco plants. Hybrid plants obtained by multiple cross-pollination events co-expressed all components and produced a functionally active secretory antibody. Earlier, complex secretory monoclonal antibodies could only be generated by extremely complicated techniques, like the *in vitro* conjugation of secreted dimeric immunoglobulin from mammalian plasma cells with the secretory component derived from epithelial cells. The secretory antibodies were found to be more stable because of their dimeric nature and thus protected teeth from infection for a longer period as compared to murine IgG. The first human trial of a monoclonal secretory antibody produced in transgenic plants was conducted by Planet Biotechnology, Inc (Mountain View, CA). Their product CaroRxTM was recombinant sIgA/G, purified from mature tobacco plants by ammonium sulphate precipitation and protein G immunoaffinity chromatography. The clinical efficacy of both plant sIgA/G and murine IgG monoclonal antibodies was tested by their application directly to teeth for three weeks with two applications per week. In both cases there was no recolonization by *Streptococcus mutans* in saliva or teeth till day 118 of the experiment^{43,44}.

Several other antibodies and antibody-derived fragments of therapeutic and diagnostic value have been expressed in plants. A few examples are cited in Table 2. There are different strategies adopted by different workers for the expression of complete antibodies in plants. This requires the expression of two genes in transgenic systems resulting in the synthesis of two proteins and subsequent correct

assembly of tetrameric protein into the functional antibody. For production of complete antibodies in plants to a level of 0.055 to 5% of the total cellular protein, following three strategies have commonly been applied.

1. Separate transgenic plants are obtained with individual heavy and light chain expressing vectors. Plants expressing high level of functional antibody are produced in the progeny after cross-pollination between plants containing heavy/light chain³⁶.
2. As plant expression vectors are generally large and contain only one promoter and one polylinker region, one vector each is used for every immunoglobulin gene. Therefore, two separate vectors, one encoding heavy chain and the other encoding light chain, are used to obtain a transgenic plant. The level of expression achieved is generally low⁵⁶.
3. A single vector with genes encoding both the heavy and light chains is used to obtain a transgenic plant⁵⁷.

Production of Pharmaceutically Important Proteins in Plants

In recent years, considerable attention has been paid to the production of human proteins in plants. Unlike bacteria, plants perform many of the complex protein-processing steps required to produce mammalian proteins in an active form. Several groups have successfully transferred the genes for the production of mammalian proteins, including human serum albumin, human α -interferon, human erythropoietin etc, in transgenic plants or cultured plant cells (Table 3).

Glucocerebrosidase (GC) catalyses the degradation of complex glycosylceramide lipids in humans. Inherited deficiency in GC causes Gaucher's disease and leads to bone marrow expansion, bony deterioration and visceromegaly. Enzyme replacement therapy of this disease involves regular intravenous administration of specifically modified human GC (ceredase). Ceredase is one of the most expensive drugs of the world. For a 50 kg person, the drug alone currently costs in the range of \$ 70,000 to \$ 300,000 per year. Successful production of human GC (hGC) in plants can clearly denote a dramatic example of the potential of plant-based system for the cost-effective bioproduction of human pharmaceutical proteins. The gene of hGC was engineered into tobacco using

Table 2—Expression of certain antibodies and antibody-derived fragments of therapeutic and diagnostic value in plants

Target	Antibody and antibody derived fragments	Plant	Reference
<i>Streptococcus mutans</i>	Guy's 13 in 3 forms: Plant G13: Guy's 13 IgG1 Plant G1/A: Guy's 13 IgG/IgA hybrid heavy chain consisting of variable $\gamma 1-\alpha 2-\alpha 3$ domains Plant G2/A: Guy's 13 IgG/IgA hybrid heavy chain consisting of variable $\gamma 1-\gamma 2 \alpha 2-\alpha 3$ domains	Tobacco	Ma <i>et al</i> ⁴⁵
Human creatine kinase	MAK33 IgG and Fab fragment	<i>Arabidopsis thaliana</i>	De Wilde <i>et al</i> ⁴⁶
CD-40, Non Hodgkin's lymphoma	Single chain immunotoxin composed of bryodin 1 fused to scFv region of anti-CD 40 antibody G28-5	Tobacco	Francisco <i>et al</i> ⁴⁷
Herpes Simplex Virus (HSV)	Humanized anti-herpes simplex virus 2 (HSV-2) monoclonal antibody (IgG)	Soybean	Zeitlin <i>et al</i> ⁴⁸
Non Hodgkin's lymphoma	scFv of IgG from mouse B cell lymphoma	Tobacco	McCormick <i>et al</i> ⁴⁹
Zearalenone produced by members of <i>Fusarium</i>	scFv	<i>Arabidopsis thaliana</i>	Yuan <i>et al</i> ⁵⁰
Carcinoembryonic antigen (CEA)	scFv Ab(scFvT84.66)	Wheat and rice	Stoger <i>et al</i> ⁵¹
Carcinoembryonic antigen (CEA)	T84.66/GS8 diabody	Tobacco	Vaquero <i>et al</i> ⁵²
Hepatitis B surface antigen	scFv	Tobacco	Ramirez <i>et al</i> ⁵³
Human anti-rhesus D	IgG1	<i>Arabidopsis thaliana</i>	Bouquin <i>et al</i> ⁵⁴
Human chorionic gonadotropin (hCG)	scFv, diabodies and chimeric antibodies (mouse variable domain and human immunoglobulin constant domain)	Tobacco	Kathuria <i>et al</i> ⁵⁵

Agrobacterium-mediated transformation. Such plant-produced hGC was found to be enzymatically active, which indicated that the gene product was correctly folded in the plant cell. This result represents one of the first active human enzymes produced in transgenic plants. Further, in the crude extract of transgenic tobacco leaves, hGC represents 10% of the total soluble protein⁶⁶. At this expression level, one tobacco plant could yield sufficient enzyme for at least one standard therapeutic dose. For the ease of growing plant biomass, plant based bioproduction of recombinant hGC should yield safe (human pathogen free) protein at a significantly lower cost than that available through existing technologies.

Collagen is found in all connective tissues and represents 30% of total body proteins in mammals. Collagen I is widely exploited for medical use, cosmetics, therapeutics and also as gelatin in food. Tobacco plants were transformed with cDNA

encoding the human prepro $\alpha 1$ chain of collagen I via *Agrobacterium*-mediated transformation⁸⁷. Selected plants, where collagen expression level was estimated to be more than 10 mg/100 gm powdered plants, were grown to maturity and recombinant collagen was extracted and purified on the basis of its physical and chemical properties. Hydroxylation of proline residues seemed to be critical for the formation and stability of the helix. Amino acid analysis showed that recombinant collagen I expressed in transgenic plants was not hydroxylated, indicating that plants did not contain all the specific post-translational machinery necessary for collagen assembly. Nevertheless, the spectrum obtained by circular dichroism for the recombinant protein was typical of a triple helix. The recombinant collagen was resistant to trypsin at room temperature, indicating the stability of the triple helix. It had reduced thermal stability and melted at 30.5°C instead of 41.5°C. Later, however, it was shown that

Table 3—Expression of products of therapeutic and industrial importance in plants

Enzyme/protein	Host plant	Application	Reference
Human enkephalins	<i>Arabidopsis</i> and oil seed rape	Antihyperanalgesic by opiate activity	Vandekerckhove <i>et al</i> ⁵⁸
Human serum albumin	Potato	Treatment of Liver cirrhosis	Sijmon <i>et al</i> ⁵⁹
Angiotensin-I converting enzyme inhibitor peptide (ACEI)	Tomato and Tobacco	Hypertension treatment	Hamamoto <i>et al</i> ⁶⁰
Human epidermal growth factor	Tobacco	Wound repair/control of cell proliferation	Higo <i>et al</i> ⁶¹
α -Trichosanthin	<i>Nicotiana benthamiana</i>	Ribosome inactivating protein	Kumagai <i>et al</i> ⁶²
	Tobacco		Krishnan <i>et al</i> ⁶³
Hirudin	<i>Arabidopsis</i>	Treatment of thrombosis	Parmenter <i>et al</i> ⁶⁴
Erythropoietin	Tobacco	Anemia	Matsumoto <i>et al</i> ⁶⁵
Protein C (hPc)	Tobacco	Highly processed serum protease of coagulation/anticoagulation cascade. Used in replacement therapy with Gaucher's disease	Cramer <i>et al</i> ⁶⁶
Human hemoglobin	Tobacco	Blood substitute	Diercyk <i>et al</i> ⁶⁷
Bryodin 1	Tobacco	Inhibition of protein synthesis	Francisco <i>et al</i> ⁶⁷
Human β casein	Potato	Baby food and for prevention of gastric and intestinal diseases in children	Chong <i>et al</i> ⁶⁸
Murine GM-CSF (Granulocyte-macrophage colony stimulating factor)	Tobacco	Neutropenia	Lee <i>et al</i> ⁶⁹
Human GM-CSF			James <i>et al</i> ⁷⁰
Lactoferrin	Tobacco	Antimicrobial properties	Salmon <i>et al</i> ⁷¹
	Potato		Chong <i>et al</i> ⁷²
	Rice		Humphrey <i>et al</i> ⁷³
Human Interleukin-2 (IL-2) and Interleukin-4 (IL-4)	Tobacco	Clinical diagnostics	Magnuson <i>et al</i> ⁷⁴
β -Glucuronidase	Corn	Diagnostic	Kusnadi <i>et al</i> ⁷⁵
α -Lactalbumin (human)	Tobacco	Milk protein with good digestibility	Takase & Hagiwara ⁷⁶
α -Lactalbumin (synthetic porcine)	Maize		Yang <i>et al</i> ⁷⁷
Preproricin	Tobacco	Protein toxin with therapeutic application in many diseases and in disease model system involving apoptosis.	Sehnke & Ferl ⁷⁸
Aprotinin	Maize	Serine protease inhibitor for transplantation surgery	Zhong <i>et al</i> ⁷⁹

Contd—

Table 3—Expression of products of therapeutic and industrial importance in plants—*Contd*

Enzyme/protein	Host plant	Application	Reference
Avidin	Maize	Biopesticide against a spectrum of insect pests, biotin binding protein used in several diagnostics	Kramer <i>et al</i> ⁸⁰
	Tobacco		Burgess <i>et al</i> ⁸¹
Human somatotrophin	Tobacco chloroplasts	Therapeutic	Staub <i>et al</i> ⁸²
Human acetylcholinesterase	Tomato	Diagnostic and therapeutic	Mor <i>et al</i> ⁸³
Human interferon - α 2b and 8	Potato	Diagnostic and therapeutic	Ohya <i>et al</i> ⁸⁴
SMAP-29 peptide	Tobacco	Antimicrobial peptide of innate immunity	Morassutti <i>et al</i> ⁸⁵
Avidin and streptavidin	Tobacco	Diagnostic	Murray <i>et al</i> ⁸⁶

hydroxylated homotrimeric collagen could possibly be produced in tobacco plants that were co-transformed with a human type I collagen and a chimeric proline 4-hydroxylase (P4H)⁸⁸. By this approach, thus, hydroxylated collagen could be obtained with hydroxyproline level up to 8.4%. This is slightly less than 10% hydroxyproline found in native human collagen.

The dragline silk of spider, *Nepphila clavipes* has remarkable tensile strength and high elasticity. Thus, making it useful for industrial and medical purposes. Synthetic spidroin genes matching to a considerable extent to the natural spidroin sequence of MaSP1 was constructed and used to transform tobacco and potato via *Agrobacterium*-mediated transformation⁸⁹. The average expression of recombinant spider silk proteins was found to be >0.5% of the total soluble protein in transgenic plants. The plant with highest expression accumulated spider silk proteins to >2% of the total soluble protein.

Transgenic Plants with Enhanced Nutritive Value

The plastids of higher plants synthesize numerous useful compounds of nutritional value like β -carotene (provitamin A) and tocopherols (vitamin E), in addition to performing normal functions of photosynthesis. Dietary β -carotene is converted into vitamin A, which plays an important role in the normal development of humans, quenches free radicals, prevents cellular damage and supports human immune system. On the other hand, insufficient dietary vitamin A leads to eye disease, xerophthalmia. For this reason, it would be desirable to meet the daily requirements of vitamin A and this goal can be achieved by raising carotenoid levels

within staple food through genetic engineering. In β -carotene biosynthesis, four specific enzymes are involved and phytoene synthase is the first and key enzyme in this pathway. The phytoene synthase gene from Daffodil was expressed in rice⁹⁰. Thus, it was demonstrated in plants for the first time that, in principle, it is possible to engineer a critical step in non-photosynthetic and carotene deficient tissue. Further, the phytoene synthase gene *crtB* from *Erwinia uredovora* was overexpressed in *Brassica napus* in a seed-specific manner, using a seed-specific promoter of *napin* gene from *Brassica*, in conjunction with a plastid targeting sequence from small subunit (SSU) of ribulose biphosphate carboxylase⁹¹. The embryos from the transgenic plants were orange and the total carotenoid concentration was found increased up to 50-fold in these transgenic seeds. The main carotenoids produced were α and β carotene and to a lesser extent phytoene was also present. Rice normally contains neither β carotene nor any of its immediate precursors but immature rice endosperm is capable of synthesizing carotenoid precursor geranyl geranyl diphosphate (GGPP). Therefore, for conversion of GGPP to β carotene, β carotene pathway was introduced into the rice endosperm via *Agrobacterium*-mediated transformation⁹². The daffodil *psy* gene, encoding phytoene synthase, and *crtI* gene from *Erwinia uredovora*, encoding phytoene desaturase, were cloned in a construct that did not have a selectable marker. Simultaneously, the gene for lycopene β -cyclase (*lcy*) and selectable marker gene were introduced using another construct. The seeds from the transgenic plants were able to produce β carotene, lutein and zeaxanthine. The transformed endosperms in most cases were yellow indicating

carotenoid formation. 'Golden rice' produced in this way is so far available as a series of pro-vitamin A producing rice (variety Taipei 309) lines. The carotenoid content of tomato fruit was enhanced by producing transgenic lines containing a bacterial carotenoid gene (*cr1*) encoding phytoene desaturase, which converted phytoene to lycopene⁹³. Expression of this gene did not increase total carotenoid levels but it increased β carotene level up to 45% of the total carotenoid content.

The lipid-soluble antioxidant vitamin E is most important to human health. Vitamin E in excess of the RDA (Recommended Daily Allowance, 100-1000 IU) is associated with decreased risk of cardiovascular disease, certain cancers and improved immune function. To achieve desired therapeutic level of this vitamin, it is important to obtain substantial increase in the tocopherol content of the major food crops. Recently, α -tocopherol (vitamin E) level was elevated in *Arabidopsis* seeds by genetic engineering⁹⁴. The last enzyme in the pathway of α -tocopherol synthesis, α -tocopherol methyl transferase was expressed in *Arabidopsis* seeds to enhance the synthesis of tocopherol.

To counteract iron deficiency that afflicts 30% of the world population, Goto *et al*⁹⁵ suggested that it may be possible to create 'ferritin rice' as an iron supplement to the human diet. They expressed the soybean ferritin gene in rice seeds, using the promoter of rice glutelin gene *GLUB-1*. This led to a high level of protein expression (0.01-0.3% of total protein) in the endosperm. The transgenic seeds accumulated 3-fold more iron than the wild type seeds.

The seed albumin gene *AMA1* from *Amaranthus hypochondriacus* was expressed in potato⁹⁶. The *AMA1* protein was non-allergenic and rich in all essential amino acids, including lysine, tryptophan, tyrosine and sulphur-containing amino acids. However, lysine, tyrosine, methionine and cysteine are the limiting essential amino acids in potato. Expression of the *AMA1* gene in transgenic tubers resulted in a significant increase in most essential amino acids. The transgenic plants also contained more total protein as compared to the wild type control plants.

A yeast gene, encoding S-adenosylmethionine decarboxylase (γ SAMdc; *SPE 2*) fused to fruit-specific and ripening-inducible E_8 promoter, was expressed in tomato, which led to an increase in the level of polyamines spermidine and spermine⁹⁷. This enhanced level of polyamines led to an increase in

lycopene content of tomatoes, prolonged vine life and better fruit juice quality.

Production of Novel Carbohydrates

Starch in many plant tissues is used as food for animals and humans. With the wealth of existing technology, it has become possible to produce altered-form of starch with unique rheologic properties or novel carbohydrate with a high cash value. Oakes *et al*⁹⁸ were the pioneers to modify storage carbon to produce new, potentially valuable product cyclodextrins (CDs). CDs are cyclic oligosaccharides containing six to eight glucose molecules. They are capable of forming inclusion complexes with hydrophobic substances. CDs are used in various applications, such as pharmaceutical delivery systems, flavour/odour enhancement and removal of undesired compounds, e.g. caffeine from food. However, cyclodextrin glycosyltransferase (CGT), which produces CD from starch, is found only in bacteria, e.g. *Bacillus* sp., *Klebsiella pneumoniae* etc. Potato was chosen as target plant because it accumulates starch in tubers and is easy to transform. The *cgt* gene from *Klebsiella* was put under the control of patatin promoter, specific to tubers. CGT accounted for 0.01% of the total cellular protein. Reasons for this low-level expression could be poor mRNA stability and translatability. It is also possible that CGT enzyme could become trapped in starch granule during development and was unable to interact with all substrate molecules. Potato tubers contained 14% starch and of this only 0.001 to 1.0% were converted to CD.

During plant growth, cellulose microfibrils give tensile strength to cell walls and lignin imparts rigidity. As an industrial viewpoint, there has been a desire to develop healthy trees that accumulate less lignin to facilitate pulping. Transgenic *Populus tremuloides* trees were produced, in which the expression of *Pt4CLI* gene encoding 4-coumarate: coenzyme A ligase (4CL) was down regulated by antisense inhibition⁹⁹. This gene product is essential in the lignin biosynthetic pathway. The transgenic trees exhibited up to 45% reduction of lignin, which was compensated for by a 15% increase in cellulose. Also, the structural integrity remained intact at the cellular and whole plant levels.

Inulin consists of linear β (2 \rightarrow 1) linked fructose chains attached to a sucrose molecule. Inulin influences the micro-flora of the gut and has beneficial effects on mineral absorption, blood lipid

composition and prevention of colon cancer. Transgenic potato plants were created that constitutively expressed *1-SST* (sucrose: sucrose 1-fructosyltransferase)¹⁰⁰, which catalyses the synthesis of trisaccharide 1-ketose from two molecules of sucrose, thereby releasing glucose and 1-*FFT* (fructan: fructan 1-fructosyltransferase), which in subsequent steps transfers fructosyl residues from one fructan to another producing fructans of different chain lengths. Inulin was made up to 5% of dry weight of transgenic tuber.

Starch in most crops contains 20-30% amylose and 70-80% amylopectin. The ratio of these two components greatly influences the physicochemical properties of starch. The properties of starch were altered by changing levels of starch synthases (SS) and starch branching enzyme through genetic modification¹⁰¹. Transgenic potato plants with greatly reduced levels of starch branching enzymes A and B were produced by *Agrobacterium*-mediated retransformation of antisense *SBEB* (starch branching enzyme B) line with partial length *SBEA* (starch branching enzyme A) construct. Starch from these lines had amylose content of 60-89%. Another much desired property of starch is freeze-thaw stability of gelatinised starch paste for use by the food industry in products that are frozen. An approach to create freeze-thaw stable starch could be the modification of starch structure by removing long chains of amylose and reducing the branch chain length of amylopectin. This type of starch was created by antisense down regulation of three starch synthase genes, viz. GBSS (granule bound starch synthase), starch synthase I (SSI) and starch synthase III (SSIII)¹⁰². Inhibition of GBSS leads to the production of amylose free starch; whereas, inhibition of SSI and SSIII leads to alteration in branch chain length distribution of amylopectin. The freeze-thaw stability of this starch remained good even after five freeze thaw cycles.

Production of Enzymes in Plants

α -Amylase

This enzyme from bacteria and fungi finds use in starch processing, alcohol production, increasing bread volume, clarifying wines and juices and in detergent manufacture. The enzyme α -amylase from *Bacillus licheniformis* is most commonly used for starch liquefaction. The enzyme is suited because of its heat stability and its activity over a wide pH range. Tobacco has been transformed with the gene for α -amylase from *B. licheniformis*¹⁰³. The enzyme

expressed at 0.3% of the total soluble protein. The biological activity did not differ from the bacterial counterpart of the enzyme. The nature and the quality of the products obtained from corn and potato starch with the help of plant-based α -amylase indicated that the enzyme produced in tobacco is well suited for the liquefaction of starch. Direct fructose production was engineered in transgenic potato tubers created by using a fusion gene encoding two thermostable enzymes, viz. α -amylase (from *Bacillus stearothermophilus*) and glucose isomerase (from *Thermus thermophilus*), which were placed under the control of granule-bound-starch synthase promoter¹⁰⁴. More than 100 independent transgenic lines were generated. This enzyme complex was active only at high temperature. When crushed transgenic tubers were heated for 45 min at 65°C, starch was degraded to produce glucose and fructose. Biochemical analysis showed an increase in the formation rate of fructose and glucose by a factor of 16.4 and 5.7, respectively in transgenic tubers as compared to wild type untransformed tubers.

Xylanase

Plant cell walls are made of proteins, cellulose, hemicellulose and lignin. Xylan is a heteropolymer of pentose sugar found in secondary walls and is a major component of cell wall in mature woody tissues. Xylanases, enzymes involved in the hydrolysis of xylan at high temperature, are important for paper pulp industry, agriculture, food industry and processing of plant fibres, like flax, hemp etc. In general, the cell wall degrading enzymes have so far only been generated with bacterial culture. Recently, gene for xylanase (*xynZ*) from *Clostridium thermocellum* was introduced into tobacco in a way that fulfils the criteria of (a) high level expression, (b) high stability and activity of the enzyme, and (c) easy purification¹⁰⁵. Now attempts are being made to create plant bioreactors for hydrolytic enzymes with high specificity for specific cell wall components.

Phytase

Phytate is the main storage form of phosphorus in many plant seeds and in this form is a poor nutrient for monogastric animals. It is routine in livestock farming to supplement diet with phytase (enzyme which release phosphates from the substrate) to improve digestibility and nutrient utilisation. Addition of phytase to feed has been shown to optimise phosphorus utilisation and to reduce excretion of phosphate in the manure of pigs and poultry. The

abundance of phosphate in the environment causes eutrophication of surface water. A reduction in phosphorus in excretion by application of phytase will, thus, have a favourable impact on the environment. Tobacco plants have been transformed with phytase gene from *Aspergillus niger*¹⁰⁶. The maximum level of the enzyme synthesized was 1% of soluble protein in seeds. The enzyme was found to be more stable in seeds. Further, addition of milled transgenic phytase containing seeds to animal fodder stimulated the release of inorganic phosphate in the digestive tract of poultry, demonstrating that phytase formulated in seeds exhibited the desired activity on plant phytate. Recently, transgenic seeds of phytase producing canola have been directly used in animal feed (PhytaSeedTM, Gist-brocades NV, Delft, Netherlands)^{44,107}.

Biodegradable Plastics

In response to the problems associated with plastic waste and its effect on the ecosystem, there has been immense focus on the development and production of biodegradable plastics. Degradable plastics can be classified either as photodegradable or biodegradable. Photo-degradation leads to breakdown of the polymer into smaller non-degradable fragments. On the other hand, biodegradable polymer can be degraded by non-enzymatic hydrolysis or by the action of enzyme secreted by microorganisms. Among the biodegradable plastics available, there is much interest in the group of polyhydroxyalkanoates (PHAs) polymers, produced entirely from bacterial fermentation. However, disadvantages in microbial production *via* fermentation include the high cost and low production. With the recent advances in molecular biology and genetic engineering allowing the possibility of using crop plants, the PHA biosynthetic genes from a bacterium (*Alcaligenes eutropus*) were expressed in *Arabidopsis thaliana*¹⁰⁸. The transgenic *A. thaliana* produced low level of PHA (100 µg/g fresh weight); however, the suspension cell culture derived from it produced 500 µg PHA/g fresh weight. To overcome the problem of low yield, the biosynthesis of PHA was targeted into the plastid and a level of 10 mg/g fresh weight was achieved. Thus, redirecting the PHA biosynthetic pathway from the cytosol to the plastid resulted in a 100-fold enhancement in PHA production¹⁰⁹. Further, the constitutive chloroplast localized expression of *phbA* (beta-ketothiolase) gene lead to again a very low production of transgenic PHB (polyhydroxybutyrate)¹¹⁰; the expression of PHB

affected the growth and development of the transgenic plant. Using a somatically activated promoter, PHB accumulated up to 3.2 mg per g dry weight. This involved the insertion of Ac element between the CaMV 35S promoter and the coding region of the β-ketothiolase gene. This strategy would allow the somatic activation of expression of β-ketothiolase gene only after the Ac element has been excised due to establishment of the link between the promoter and coding sequence. Another strategy employed was the use of *PRP-1* promoter from potato, which could be induced by wounding, or application of salicylic acid. Using this construct, maximal amount of PHB accumulation was found to be 420 µg per g dry weight. However, high expression of PHB affected growth and development of transgenic plants. Phasins are a class of proteins known from PHB-producing bacteria, which serve as protectants against highly hydrophobic surface of the PHB granules within the bacterial cell. When the phasin gene was co-expressed with the PHB biosynthesis genes, the symptoms, however, were not reduced. Thus production of PHB would require further efforts to overcome the problems faced.

The world consumes approximately 93% of fossil resources for energy production, while only 7% are used for the production of a variety of organic chemicals, including solvents and plastics. Replacement of a fraction of synthetic plastics with biodegradable polymer produced from renewable resources is, thus, likely to have a small impact on the overall consumption of fossil fuel. Nevertheless, greater use of biodegradable plastics could significantly contribute to help solve problems associated with environmental pollution and waste management.

Conclusions

The findings to date indicate that plants can be exploited as bioreactors and cost-effective alternative to microbial and animal systems for the production of biomolecules. The pharmaceutical products from animal sources are feared for the possible association with microbes, which could be pathogenic to humans. For example, human serum albumin with a worldwide demand of approximately 550 metric tonnes of purified protein per year is isolated conventionally from human blood donations, and is therefore, expensive. Besides, it also bears the risk of virus contamination. Plants, therefore, offer a safer system for the production of human serum albumin and other

such products. Some transgenic products like maize avidin (Sigma-Aldrich, St Louis, MO), β -glucuronidase (Prodigene Inc., College Station, TX) already occupy the market; others are likely to be released soon. It is, however, possible that the effect of some biomolecules on plant health may come in the way of viable production systems. In view of this, each case would have to be tested experimentally. With the dramatic development of biotechnology, the days are counted to have a designer food product for breakfast, which can provide required nutrients, energy and protection for us.

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