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Lesson: Role of Plant Biotechnology in Industry

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Chapter: Role of Plant Biotechnology in Industry

Introduction

Plant biotechnology is among the newer tools used for maximizing the potential of agriculture and for the benefit of society in a large number of other ways. Plants can be exploited as bioreactors for production of economically viable recombinant biomolecules. Increased production of a chemical within a plant may also be cost effective as compared to other methods like fermentation. Plants have emerged as convenient, eco-friendly and economical alternatives over other expression systems. This is also referred to as molecular farming wherein genetically modified plants are being used for the production of various significant pharmaceutical, therapeutic or other industrial products. It has the potential to provide large amounts of supply of recombinant proteins providing a lucrative alternative to other conventional protein production methods.

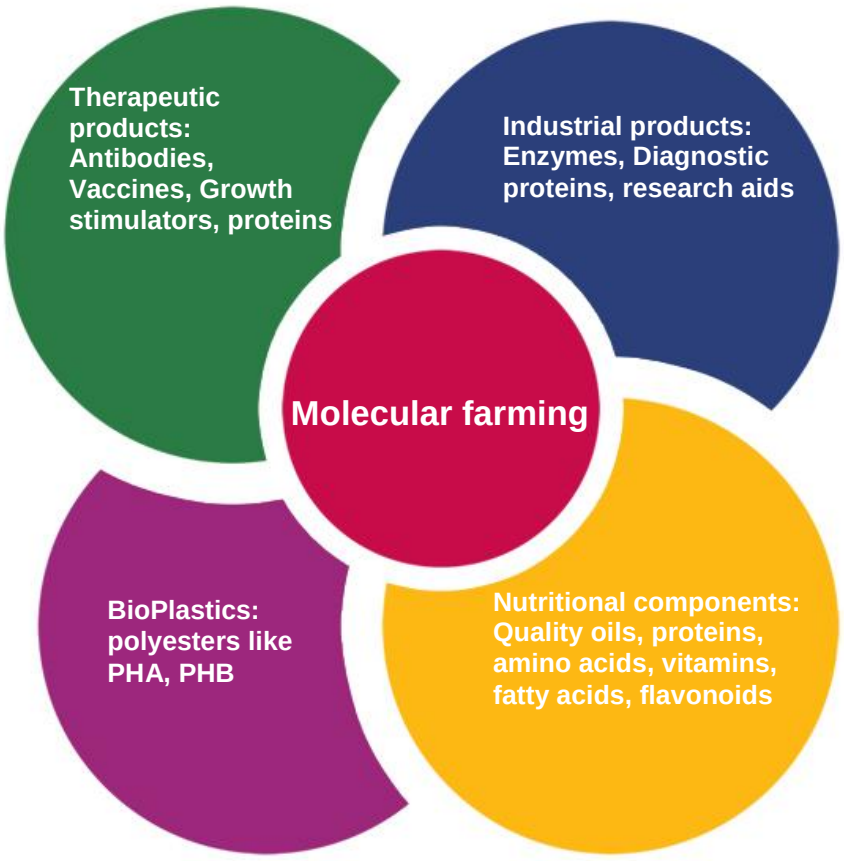

Figure: Molecular farming is used for the production of various therapeutic products, nutritional components, bioplastics and other industrial products.
Source: Author

In recent years, several proteins and biomolecules have been produced in genetically modified plants by the introduction of foreign genes. The inserted gene (transgene) may be from an unrelated plant or from a completely different species. These plants are thus genetically modified. There are basically two strategies for production of foreign molecules:

i) Production of transgenic plants by stable integration of a transgene in plant either using naturally occurring plasmids of *Agrobacterium* or by using direct gene transfer.

ii) Transient expression of a transgene by using vectors like plant viruses.

A widely used technique is *Agrobacterium* mediated transformation where the genes of interest is transferred into plant genome (Refer to the chapter on Methods of Gene Transfer). *A. tumefaciens* is a soil bacterium that contains Ti (tumor inducing) plasmid and causes crown gall disease in a number of dicotyledenous plants. Infection occurs when the bacterium invades a wound in the plant stem and causes cancerous proliferation in the region of the crown because of the presence of Ti plasmid. This is a large (200kb) plasmid and carries genes involved in the infection process. A part of Ti plasmid known as T-DNA gets inserted in the plant genome, is maintained stably and is passed to daughter cells. New genes can be inserted in the T-DNA and integrated in the plant genome. Another plant vector is based on Ri plasmid of *Agrobacteium rhizogenes* which causes hairy root disease in a number of dicotyledenous plants.

Biolistics i.e., bombardment with microprojectiles to introduce foreign DNA directly into plant embryos is also being used widely. In addition, techniques like electroporation, and polyethylene glycol (PEG) mediated direct gene transfer are also being employed.

While producing a transgenic plant, it is important that the transgene achieves a high level of expression. Biolistics and PEG induced direct gene transfers could also be used to transfer genes into the chloroplast genome provided the foreign DNA carries sequences similar to chloroplast genome and integration occurs via homologous recombination. Chloroplast transgenes generally result in high expression levels. This can also be achieved using suitable strong promoters.

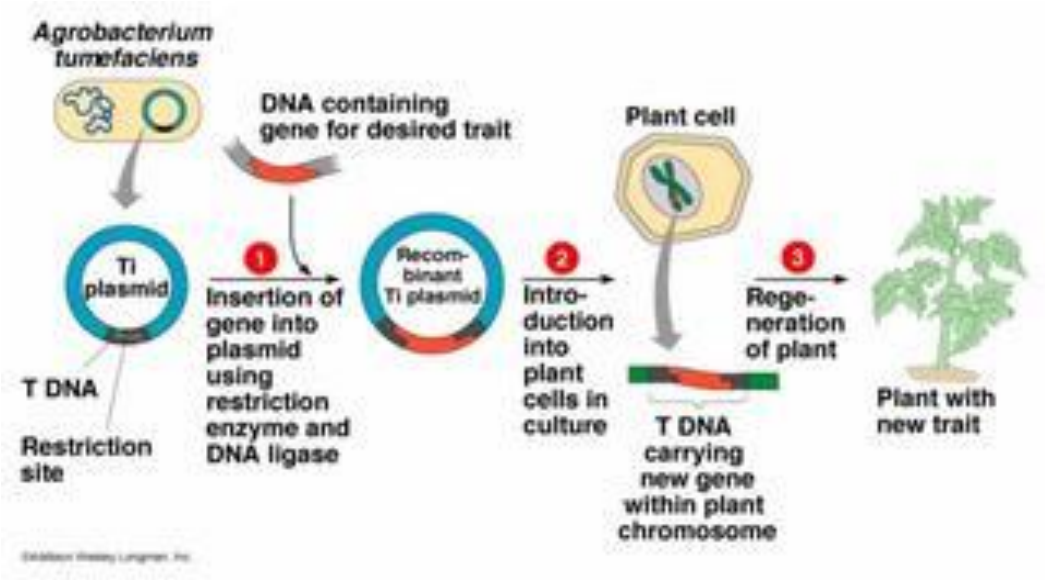

Figure: Gene of interest is first inserted in the Ti plasmid of *Agrobacterium tumefaciens.* It is then introduced into the plant cells in culture. The new gene gets integrated within the host genome. After selection the transgenic plant is regenerated.

Source:

http://buildyourownbombshelter.wikispaces.com/file/view/transgenic_plant.jpg/296431856/320x176/transgenic_plant.jpg(cc)

Link for animation of *Agrobacterium* mediated gene transfer:

http://highered.mheducation.com/sites/9834092339/student_view0/chapter17/genes_into_plants_using_the_ti-plasmid.html

Advantages

There are various advantages of using plants as bioreactors:

1. They have mechanisms of post-translational processing.
2. They are cost effective and have lower upstream production costs. They also lower storage costs.
3. It is easier, faster and less expensive to produce transgenic plants as compared to transgenic animals.
4. Plants that generate large biomass like corn, tobacco etc. are capable of producing large amounts of products.
5. Proteins can be stored in seeds for longer times with little reduction in quality.
6. Most of the plant pathogens are harmless to humans.

7. Plants generally mature in a season enabling faster marketing of plant made products, thus leading to easier scale up.

Disadvantages

Besides, these advantages there are numerous disadvantages of using plants as bioreactors:

1. Codon bias i.e., differences in codon usage between various species and between prokaryotes and plants could lead to insufficient expression of proteins in plants.
2. There may be inconsistencies in dosage of plant made products from different fruits, plants, plant tissues and generation of plants.
3. Certain allergic compounds are produced by plants.
4. Issues relating to ethical, social, environment and biosafety are still debatable.

Table: Properties of different systems used for production of recombinant proteins.

System	Overall cost	Production timescale	Scale-up capacity	Product quality	Glycosylation	Contamination risks	Storage cost
Bacteria	Low	Short	High	Low	None	Endotoxins	Moderate
Yeast	Medium	Medium	High	Medium	Incorrect	Low risk	Moderate
Mammalian cell culture	High	Long	Very low	Very high	Correct	Viruses, prions and oncogenic DNA	Expensive
Transgenic animals	High	Very long	Low	Very high	Correct	Viruses, prions and oncogenic DNA	Expensive
Plant cell cultures	Medium	Medium	High	High	Minor differences	Low risk	Moderate
Transgenic plants	Very low	Long	High	High	Minor differences	Low risk	Inexpensive

Source: http://scialert.net/qredirect.php?doi=rjmp.2012.466.488&linkid=pdf(cc)

Production of therapeutic products

Plant bioreactors are being employed for the production of various Plant derived antibodies and vaccines.

1. Plantibodies

The term plantibody (*plant* + *antibody*) is used to describe antibody or antibody fragments produced from transgenic plants. These antibodies are being produced within the plants efficiently using plants as protein factories.

Figure: A cartoon depicting Plantibodies i.e., antibodies derived from transgenic plants which are being used for therapeutic purposes.
Source: http://image.slidesharecdn.com/plantibodiesppt-131118235833-phpapp02/95/plantibodies-1-638.jpg?cb=1384840787(cc)

Antibodies (also known as immunoglobulins) are complex glycoproteins produced within the body as a mechanism of cellular defence (immune system) that recognizes foreign antigens in response to infections. Antigen is the foreign substance that, when introduced into the body, stimulates an immune response by activating lymphocytes to produce antibodies or attack antigen directly. All antibodies have a common structure consisting of four polypeptide chains (two identical light (L) chains and two identical heavy (H) chains) forming a Y shaped molecule linked by disulphide bridges. Tip of the "Y" of an antibody has a paratope (a structure similar to a lock) specific for one particular epitope (structure analogous to a key) on an antigen, so that there is a perfect complementarity between them and they bind to each other like a lock and key. There are five major isotypes of antibodies in mammals based on heavy chains they possess: IgG, IgM, IgD, IgE and IgA. The first 110 amino acid residues within different antibodies have varying specificity and are therefore known as V (variable) regions. They occur on light and heavy chains (V_L and V_H). Relatively invariable region following V regions are known as C (constant) region. The maximum variability among antibodies occurs at residues known as the hypervariable regions or the complementarity determining regions (CDRs) within the V regions. Since the V region of both H and L chains exclusively determines antigen binding, it can be produced in plants.

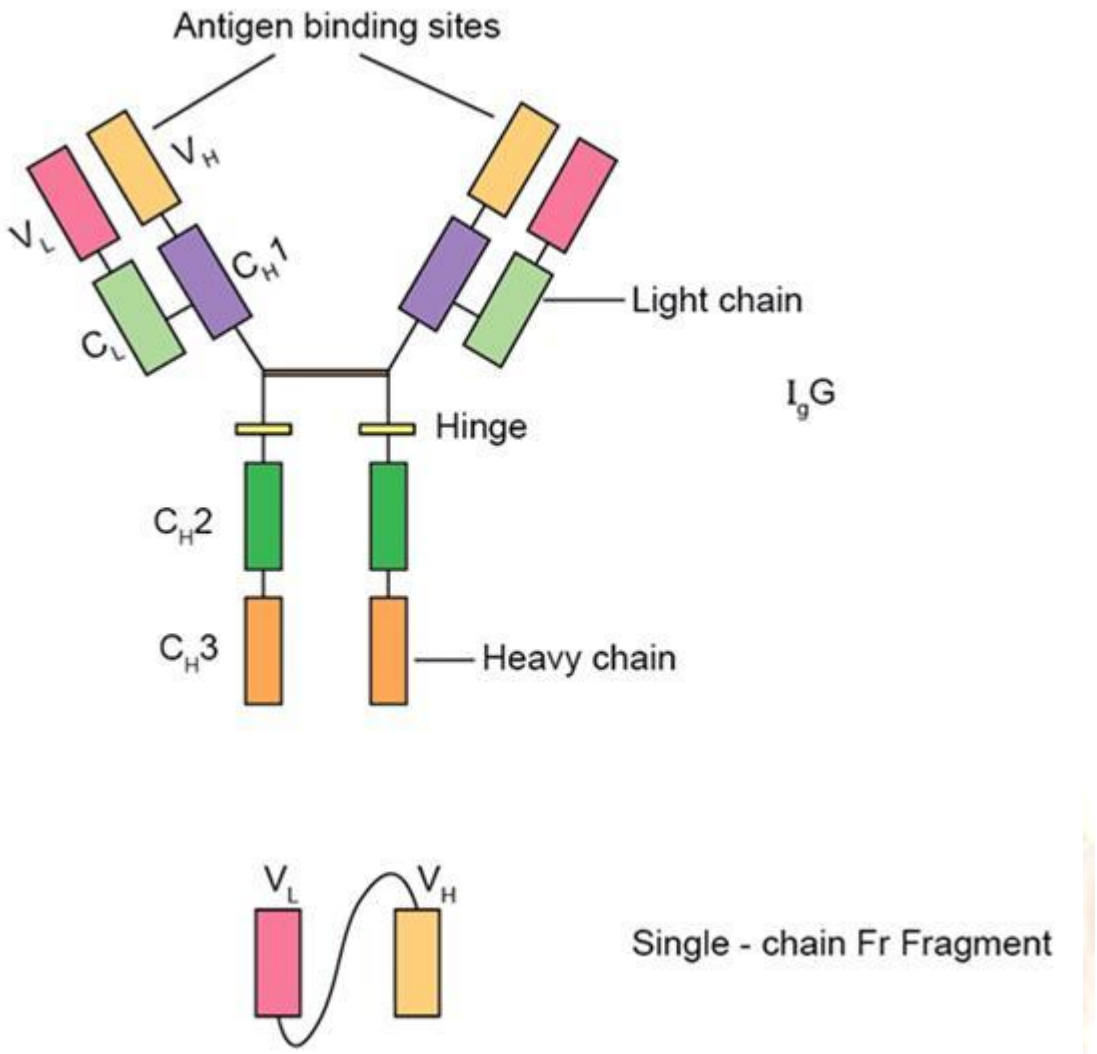

Figure: Structure of an antibody

Source: http://nptel.ac.in/courses/102103016/41(cc)

Antibodies produced by a single clone of cells are known as monoclonal antibodies. They are monospecific antibodies i.e., have affinity for the same antigen. Therapeutically they are very useful and are being produced by costly fermentation methods. Monoclonal antibodies can also be produced using plants.

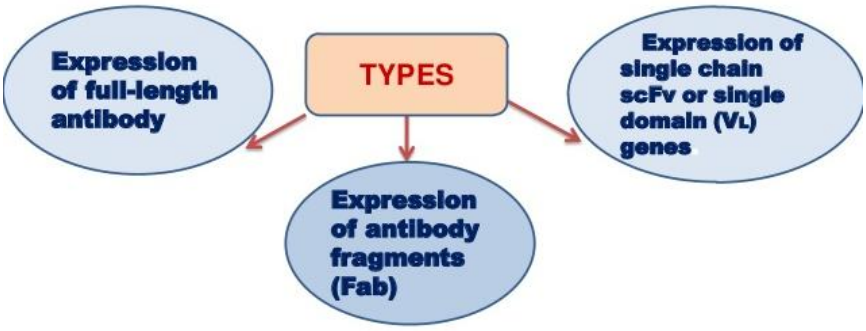

Figure: Three major types of plantibodies
Source: http://image.slidesharecdn.com/plantibodiesppt-131118235833-phpapp02/95/plantibodies-2-638.jpg?cb=1384840787

Method of plantibody production

For plantibody production, the cloning of coding sequences of heavy and light chain regions is carried out in suitable vectors. Cloning is done in two plant cell lines and for the establishment of two transgenic lines (one possessing heavy chain gene and other having light chain gene). Then these two lines are crossed to generate F1 progeny expressing both the chains from a double transgenic plant.

These plantibodies could also be engineered to be expressed in endoplasmic reticulum (for correct folding and disulphide bond formation) and could be produced in certain plant tissues like fruits, tubers, etc. which further helps to achieve higher and stable level.

Glycosylation is the addition of carbohydrate chains or glycans to proteins, lipids or other organic molecules. Plant and mammalian glycosylation patterns are different and thus need to be taken care of, for producing functional antibodies. The enzyme β-1,4-galactosyltransferase is not present in plant systems for the conversion of plant N-glycans to mammalian N-glycans. A transgenic plant expressing this enzyme could be expressed with a transgenic plant expressing heavy and light chains, which results in higher levels of optimum glycosylation.

These plantibodies are very efficient as they have identical peptide sequence to those of mammalian antibodies. These plantibodies find both *in planta* and *ex planta* applications.

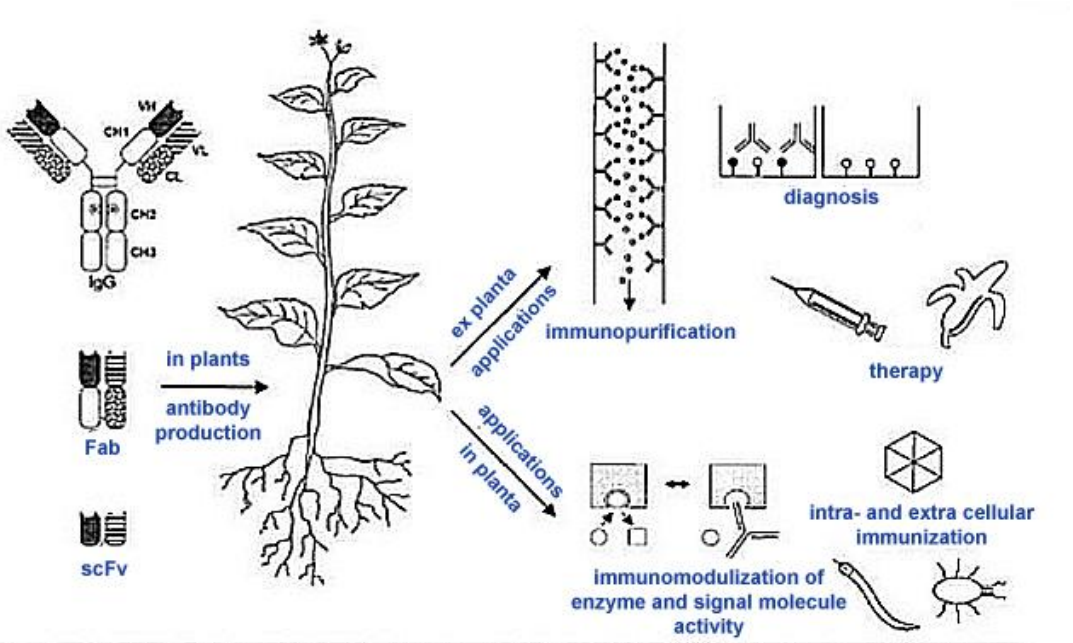

Figure: *In planta* and *ex planta* uses of Plantibodies.

Source: http://image.slidesharecdn.com/plantibodiesppt-131118235833-phpapp02/95/plantibodies-4-638.jpg

***In planta* applications of plantibodies**

In planta applications include immunomodulation of enzyme or signal molecule activity (manipulation of plant metabolism) and for intra and extracellular immunization (pathogen resistance). Immunomodulation implies to interference with cellular metabolism or pathogen infectivity by ectopic expression of antibody or fragments of antibody genes.

Plant pathogens account for major loss of crop revenues worldwide, using present control measures of crop protection. Plantibodies could be used for generation of resistant plants. It is expected that this type of plant immunization will yield results by expressing animal antibody genes in plants that will produce antibodies directed against specific antigens of plant pathogens such as virus coat proteins,fungal and bacterial enzymes of attack (Liao et al., 2006). The following table lists few examples of plantibody mediated resistance to pathogens in transgenic crops:

Table: Plantibody mediated resistance to plant pathogens				
S.No.	**Pathogen/antigen**	**Antibody**	**Target plant**	**Reference**
1.	Antichoke mottled crinckle virus	scFv	*Nicotiana tabacum*	Tavladoraki *et al.,*1993
2.	Tobacco mosaic virus	Full size	*N. tabacum, Xanthi*	Voss *et al.,* 1995
3.	Root knot nematode/stylet secretions	Full size	*N tabacum, Xanthi*	Baum *et al.,* 1996
4.	Corn stunt spiroplasma/membrane protein	scFv	*Zea mays*	Chen and Chen,1998
5.	Fusarium/ cell wall protein	scFv	*Arabidopsis thaliana*	Peschen *et al.*, 2004
6.	Beet necrotic yellow vein	scFv	*N. benthamaina*	Fecker *et al.*,1997

Source: Author

***Ex-planta* applications of plantibodies**

Ex-planta uses of plantibodies include therapy, diagnosis and affinity based purification. These plantibodies offer several advantages as therapeutic agents. They can be used for treatment of various infectious diseases, inflammation, cancer or autoimmune disorders.

Two important plantibodies

CaroRx™

CaroRx™ is the world's first clinically tested plantibody. It binds specifically to *Streptococus mutans* (etiological agent for tooth decay) and prevents bacteria from adhering to teeth. CaroRx is designed for regular topical application and allows thorough cleaning and intervention of tooth decay. It is being produced by Corporation Planet Biotechnology in transgenic tobacco plants. CaroRx™ is presently undergoing Phase II U.S. clinical trials under a U.S. FDA-approved Investigational New Drug (IND) application. Clinical trials using CaroRx™ plantibody, at Guy's Hospital, Kings College London, have shown that it can effectively remove decay-causing bacteria for up to two years. Preclinical animal studies have further corroborated its antibacterial potential.

Avicidin

Another plantibody to have reached phase II clinical trials is full-length IgG specific for EpCAM (a marker of colorectal cancer) developed as the drug Avicidin by NeoRx and Monsanto (Doram, 2000). Although Avicidin demonstrated some anti-cancer activity in patients with advanced colon and prostate cancers, it was withdrawn because it also resulted in a high incidence of diarrhoea.

They are stable, highly specific, and less toxic, could be purified easily and are cost effective. Also there is high drug approval rate and can be injected, topically applied or used orally, making plantibodies most cost effective source of antibodies. The following table list some plantibodies along with with their applications and antigens:

Table: Plantibodies and their applications

S.No.	Antibody form	Plant	Applications and antigens	Reference
1.	sIgA (CaroRx)	Tobacco (*Nicotiana tabacum)*	Therapeutic for Dental caries (Topical) Streptococcus infections (Streptococcus surface antigen)	Larrick *et al.,* 1998; Weintraub et al., 2005
2.	scFvT84.66	Wheat, Rice	Cancer; carcinoembryonic antigen (CEA)	Stoger *et al.,* 2000
3.	IgG	Soyabean	Therapeutic; Herpes Simplex Virus	Zeitlin *et al.,* 1998
4	Human IgG	Alfalfa	Diagnostic	Khoudi *et al.,* 1999
5.	IgG	Tobacco	Therapeutic; Respiratory syncytial virus	Whaley *et al.*,2011
6.	ScFv	Tobacco (*Nicotiana tabacum)*	Therapeutic; Non-Hodkins lymphoma idiotypes	McCormick, 2011
7.	Diabody	Tobacco	Therapeutic/diagnostic; carcinoembryonic antigen (CEA)	Vaquero *et al.*, 2002
8.	38C13 (ScFv)	Tobacco	Therapeutic; lymphoma	McCormick *et al.,* 1999
9.	PIPP (chimeric full-size IgG, an scFv fragment and a diabody)	Tobacco	Diagnostic; Contracception and therapeutic for tumors that produce human chorionic gonadotropin (hCG)	Kathuria *et al.*, 2002

ScFv: single chain variable fragments

sIgA: Secretory IgA

Source: Author

Progressive improvement in biotechnology can lead to development of affordable recombinant antibodies free of pathogens for human therapeutic and industrial purposes.

2. Plant based Vaccines

Traditional commercial vaccines have certain limitations despite their usefulness. They are basically either inactivated or live attenuated strains of pathogens mostly delivered intravenously or orally (oral polio vaccine). Nowadays, subunit vaccines are being used which are made of a subunit (pathogen protein or an epitope) that cannot cause a disease but is capable of eliciting an immune response. They are generally recombinant proteins produced in hosts like cultured yeast cells, which are then injected in humans to provide immunity to various diseases. They are safer than whole vaccines as they minimize the possibility of reversion and there is no need to culture the pathogen. Oral vaccines would provide better protection by stimulating both mucosal and systemic immune responses and eradicating usage of needles and medical assistance. Also dependence on cold chains for storage and transportation of vaccines could be reduced as these vaccines if expressed in for example, maize grains, would be stable at ambient temperatures and could be transported to developing and underdeveloped countries lacking refrigerated storages.

Figure: Edible corn-based vaccines produced by Prodigene, USA. These transenic corn kernels correspond to 1 mg dose of the B subunit of (a) E. Coli heat labile toxin (b) Whole corn snack (c) Fractionated corn which produces 6 times more antigens.
Source: http://scialert.net/qredirect.php?doi=rjmp.2012.466.488&linkid=pdf(cc)

Method of plant based vaccine production

Plant based vaccines are produced from transgenic plants by the introduction of desired gene encoding a specific antigen that can trigger a strong immune response inside the body. These plant based vaccines are inherently safe as they have no risk of microbiological contamination generally linked with animal-derived vaccines and at the same time reduced risk of pathogenicity, virulence reversion and shedding. Also they are cost effective and injection related hazards are also minimized. Body's first

line of defence (mucosal immunity) is elicited because of oral consumption. Their chief mode of action is stimulation of the lymphoid structure of the intestine. The figures given ahead describe the strategy to make transgenic plant vaccines.

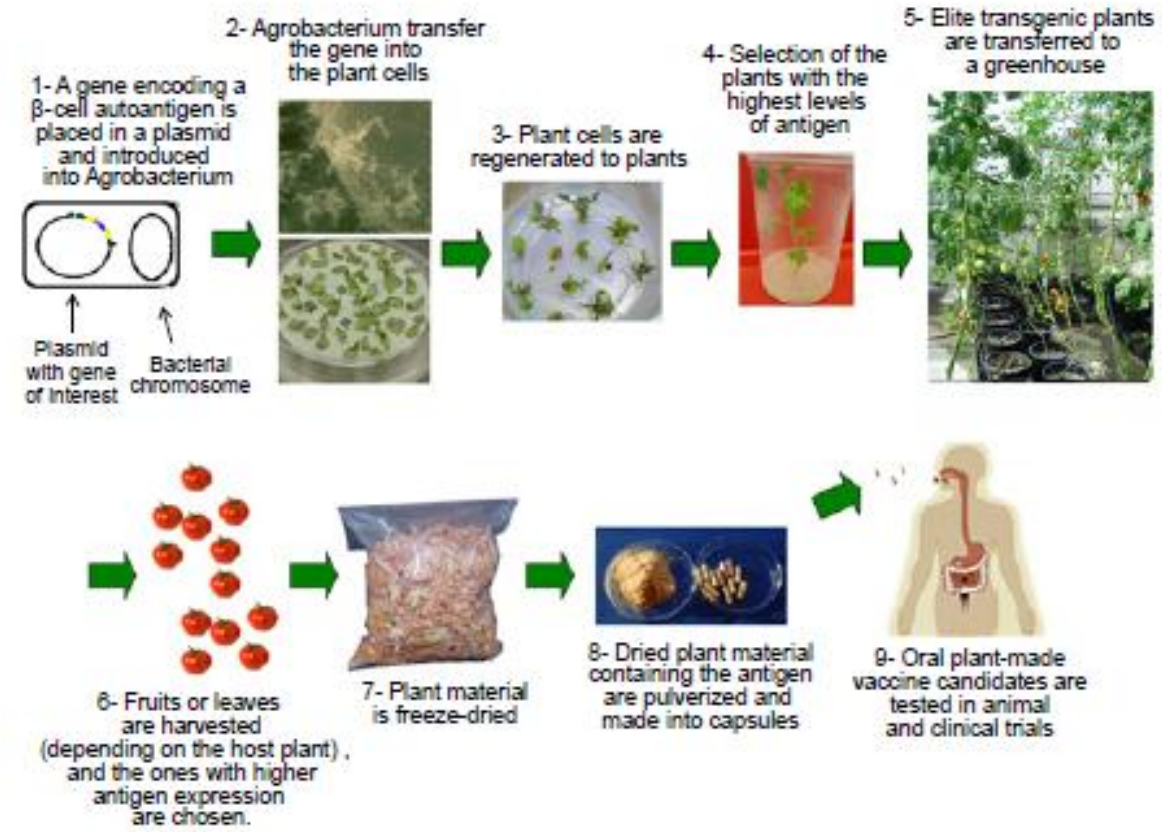

Figure: Production of a plant based vaccine or an edible vaccine

Source: http://omicsonline.org/reverse-vaccination-and-treatment-of-type-1-diabetes-using-plant-produced-autoantigens-2155-9899.S2-007.pd(cc)

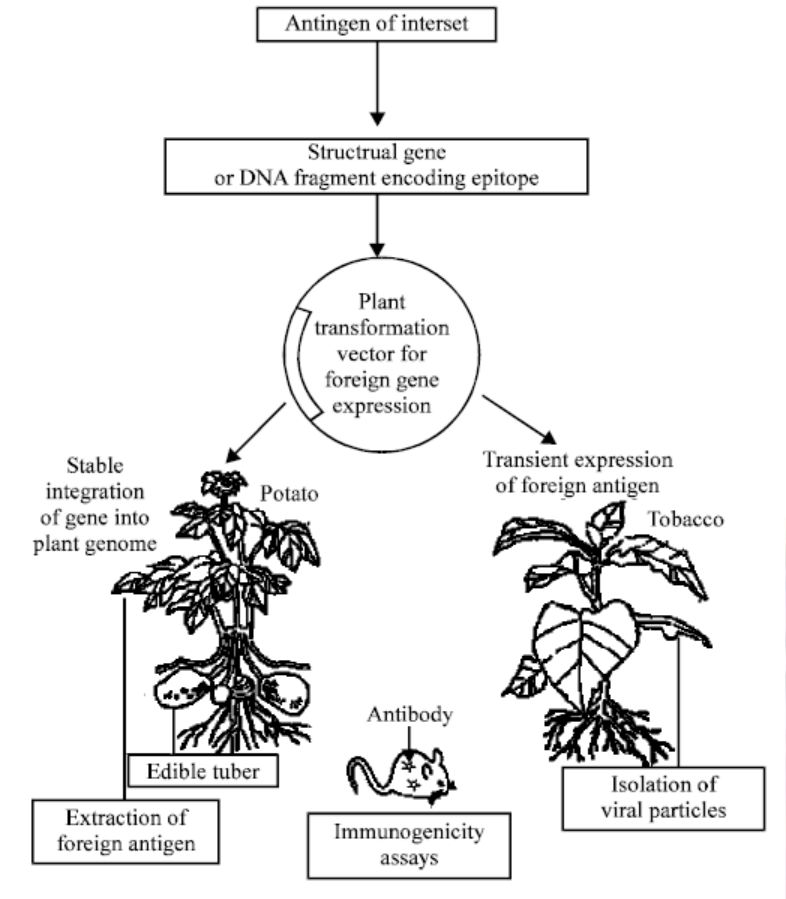

Figure: Steps in plant vaccine preparation.

Source: http://scialert.net/qredirect.php?doi=rjmp.2012.466.488&linkid=pdf(cc)

Examples of plant based vaccines

The first plant derived vaccine using the above strategy was produced in transgenic tobacco plants expressing the gene for *Streptococcus mutans* surface protein antigen A (SpaA). Its success was followed by expressing the gene for Hepatitis surface antigen. Ever since, several antigens have been expressed in plants like include *Escherichia coli* heat-labile enterotoxin antigen (LTB), cholera toxin B subunit, Enkephalins, Human serum albumin, Norwalk virus capsid protein, VP1 antigen of foot and mouth disease virus, cholera toxin B subunit, Rabies virus glycoprotein, VP6 protein of rotavirus, and an epitope from the major surface antigen of *Plasmodium falciparum* (Jain et al., 2013). A potato-based vaccine against hepatitis B has shown promising results in the first human trials. Transgenic potato expressing norwalk virus antigen showed seroconversion. Transgenic potato with CT-B gene of *Vibrio cholerae* has been shown to be efficacious in mice. Tomato plants expressing rabies antigens were also reported to induce antibodies in mice.

Rota viral disease: A case study

Rotavirus causes severe diarrhoea in infants and young children. It is a double-stranded RNA virus belonging to the family *Reoviridae*. There are five types of this virus A, B, C, D, and E; Rotavirus A being the most common cause of infections in humans. It gets transmitted through fecal-oral route.

Traditional vaccines:

A rotaviral vaccine was licensed for use in the United States in 1998. Clinical trials found it to be 80 to 100% effective at prevention of diarrhoea caused by rotavirus A. However, side effects were reported and it was withdrawn from market as early as 1999. In 2006, two new vaccines against rotavirus A infection were reported as safe and effective for children. WHO recommended compulsory vaccination against rotavirus should be included in all national vaccination programmes.

Plant vaccine: Adequate expression of exogenous antigens is a critical factor in edible vaccine development. sVP6 gene which was codon optimized coding for VP6 protein of human group A rotavirus was constructed and inserted in the alfalfa genome by *Agrobacterium*-mediated transformation. High expression levels of sVP6 (as much as 0.28% of the total soluble protein of the pBsVP6-transgenic alfalfa) was reported. Studies using immunized mice showed high titers of anti-VP6 serum IgG and mucosal IgA. Children of immunized dams developed less severe diarrhoea after infection with simian rotavirus SA-11, showing that antibodies generated in response to plant vaccine provided passive heterotypic protection to the pups. These results indicate that oral immunization might provide a potential of protecting infants from rotavirus mediated diarrhoea.

Another alternative for the production of plant based vaccines is to infect plants with recombinant virus carrying the desired antigen fused to the viral capsid protein. Plants infected with viruses having chimeric coat proteins have been reported to overproduce desired fusion protein in short intervals of time. Production platforms based upon plant viral vectors are now being employed to produce vaccines to fight various cancers, global pandemics like H1N1 influenza, and potential biowarfare agents like Ebola virus. Examples are given in the following table (Hefferon, 2012).

Table: Employment of plant virus expression vectors for vaccine production

S.no	Protein	Plant	Carrier
1.	Influenza antigen, HIV-1 antigen, Foot and mouth virus, malaria	Tobacco	TMV
2.	Rabies antigen	Spinach	AIMV

3.	HIV-1 antigen	Tobacco	AIMV
4.	Murine zona pellucida antigen	Tobacco	TMV
5.	Mink enteritis antigen	Bean	CPMV
6.	Staphlococcus Enterotoxin B (SEB)	Tobacco	Geminivirus
7.	Ebola virus antigen	*Nicotiana benthamiana*	Gemimivirus
TMV: tobacco mosaic virus CPMV: Cowpea mosaic virus AIMV: Alfalfa mosaic virus			

Source: Author

Limitations of plant based vaccines

Besides having several advantages these plant based vaccines suffer from various limitations like:

- The development of immunotolerance to the vaccine,
- Instability of vaccine and evaluation of dosage requirement.
- Also there may be inconsistencies in dose requirement from different fruits, plants, plant tissues and generation of plants.
- Sometimes selection of most suited plant is also difficult. Also those plants that cannot be consumed raw like potato need cooking that might change certain properties of the vaccine.
- They may trigger allergic responses and their longevity is debatable.

Edible vaccine banana: Scientists are designing recombinant bananas that can be used as edible vaccines against cholera and hepatitis B infections by expressing antigens of the etiological agents *Vibrio cholerae* and Hepatitis B virus. Banana doesn't need to be cooked and once eaten, would expose the body to the antigenic proteins and offer resistance later on to encounters with the infectious agents.

Biotechnology advancements and extensive research in plant based vaccines will surely remove all the drawbacks of plant based vaccines making these cost effective vaccines indispensable for human well being.

3. Other pharmaceutical products (Biopharmaceuticals)

Transgenic plants can be employed for the production of various pharmaceutically important products. Several plant–derived biopharmaceutical proteins are reaching at an advanced stage for commercial production. Few examples are cited below:

i. Recombinant Insulin (Humulin)

Humulin implies brand name for a group of biosynthetic human insulin products, made by Arthur Riggs at Genentech in 1978. Production of recombinant human insulin in *Escherichia coli* was one of the first breakthroughs of biotechnology in the pharmaceutical industry. It is widely used as a therapeutic to treat patients suffering from diabetes mellitus (DM). Figure given ahead describes production of genetically modified insulin. Cost is the limiting factor for the production of insulin.

More recently, genes for human insulin have been introduced into Safflower (*Carthamus tinctorius)* and *Arabidopsis thaliana* in order to reduce production costs. Plant derived insulin has shown to be equally stable and it accumulates in oil seeds. Their seeds are then ground, the oil extracted, and the insulin harvested. Canada based SemBioSys Genetics Inc. is commercially working on production of insulin from transgenic Safflower plants.

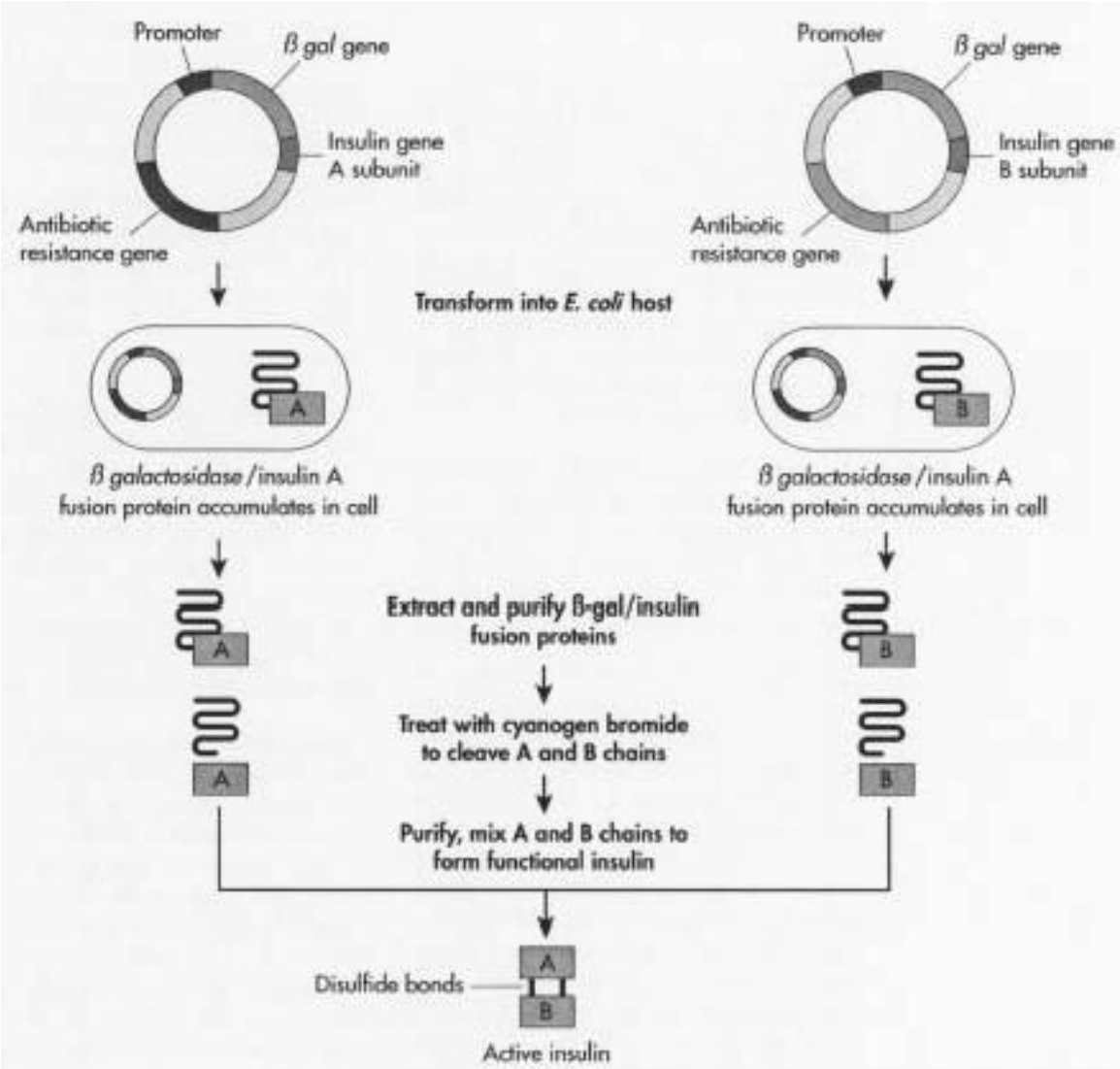

Figure: Production of recombinant insulin in *E.coli*.

Source:

http://recombinantinsulin.wikispaces.com/file/view/hpm_0000_0007_0_img0061.jpg/115684863/hpm_0000_0007_0_img0061.jpg

ii. Human growth hormone (somatotropin)

The first pharmaceutically relevant protein made in plants was human Growth Hormone (hGH), which was expressed in transgenic tobacco seeds and sunflower callus in mid eighties. Using agro-infiltration technique, scientists have transiently expressed human Growth Hormone (hGH) in tobacco (*Nicotiana tobacum*), potato (*Solanum tuberosum*) and lettuce (*Lactuca sativa*) leaves.

iii. Glucocerebrosidase

It is an enzyme localized in the lysosome and its deficiency causes Gaucher's disease. Earlier this enzyme was purified from human placenta but nowadays it is being proposed to produce it from transgenic tobacco plants. Plant derived Glucocerebrosidase has been approved commercially by FDA to use by humans.

Table: Plant-derived pharmaceuticals that are in the pipeline for commercialization

Product	Medical issue targeted	Source	State
Vaccines			
E. coli heat labile toxin	Diarrhea	Maize, potato	2 independent phase I trials
HBsAg	Hepatitis B	Lettuce, potato	2 independent phase I trials
Rabies glycoprotein	Rabies	Viral vectors in spinach	Phase I trials
Norwalk virus protein	Sickness and diarrhea	Potato	Phase I trials
Antibodies			
LSBC scFVs	Non-Hodgkin's lymphoma	Viral vectors in tobacco	At least 12 personalized antibodies submitted for phase I trials
Avicidin	Colorectal cancer	Transgenic maize	Withdrawn from phase II trials in 1998
Others			
Gastric lipase	Cystic fibrosis, pancreatitis	Transgenic maize	Phase II trials
Lactoferrin	Gastrointestinal infections	Transgenic maize	Phase II trials
Human intrinsic factor	Vitamin B12 deficiency	Transgenic *Arabidopsis*	Phase II trials

Source: http://scialert.net/qredirect.php?doi=rjmp.2012.466.488&linkid=pdf(cc)

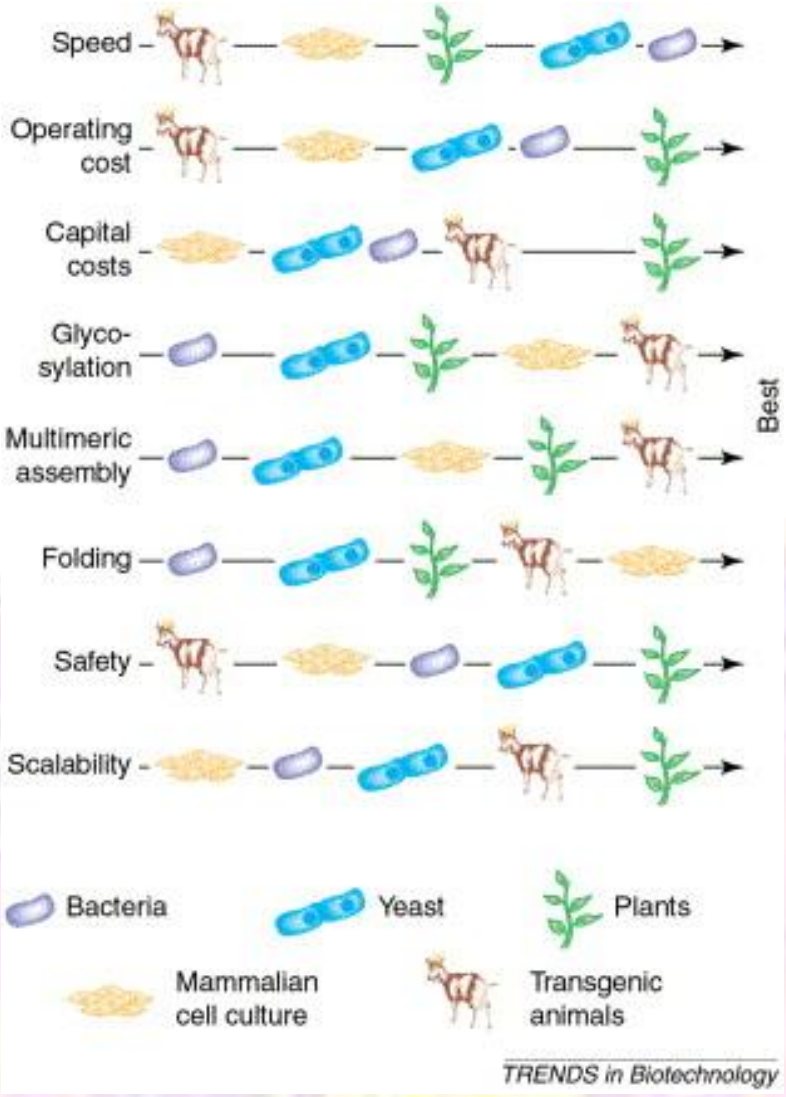

Figure: Comparison of bacterial, yeast, plant, mammalian and anima systems for production of recombinant proteins.

Source: http://www.sciencedirect.com/science/article/pii/S0167779902020802

Production of industrial products

1. Enzymes

Enzymes are biocatalysts having exceptional catalytic potential. They are mainly proteins with the exception of ribozymes. They have very high substrate specificity, accelerate rate of chemical reactions, function under optimum temperature and pH conditions in aqueous solutions and are subject to regulation making life feasible.

Presently most industrial enzymes are being produced commercially using microbial fermentation methods. An efficient and cost effective alternative approach is to use

transgenic plants as bioreactors for the production of these enzymes. It is also very tedious to produce certain enzymes using conventional recombinant systems. Avidin and β-glucuronidase (GUS) were among the first commercial proteins derived from transgenic maize plants (Hood et al., 1999). This was followed by large scale production of protease trypsin. Further high expression levels are attained using seed preferred promoters, targeting to specific subcellular locations and breeding into protective germplasm with minimum issues for plant physiology. The following table lists some important industrial enzymes produced in transgenic plants along with their applications (Beisgen et al.,2002):

Table: Industrial enzymes and their applications produced in transgenic plants

S.no	Enzyme	Applications
1.	α-amylase	Food processing, paper industry
2.	Avidin	Diagnostic kits
3.	Cellulase	Ethanol and paper industry, dish washing detergents
4.	Glucanase	Brewing industry
5.	β-glucuronidase	Diagnostic kits
6.	Trypsin	Pharmaceuticals
7.	Pepsin	Cheese production
8.	Xylanase	Biomass processing, paper and pulp industry
9.	Lignin peroxidase	Paper industry
10.	Phytase	Better phosphate utilization

Source: Author

(i) Trypsin (Protease)

It is used widely in pharmaceuticals industry, detergent additives, eye care and leather processing. It is being currently derived from bovine pancreas. Bovine slaughterhouses are bristling with occurrence of mad cow disease and foot and mouth disease. Therefore, usage of non animal sources of trypsin like transgenic plants is highly recommended.

It is very tedious to produce functional trypsin in heterologous systems as it pose a threat of degrading native proteins being a protease. Such proteins that can harm cell parts can be expressed using Seed specific promoters. In 2000, ProdiGene was issued a broad-based patent (USP # 6,087,558) describing the production of proteases in transgenic plants, claiming expression of any protease in transgenic plants, in its zymogenic form (inactive precursor form). Higher expression levels are

obtained using constitutive and seed specific promoters for trypsinogen (zymogen) gene as compared to active trypsin gene Currently, for corn-derived trypsin, the product TrypZean™ is a lyophilized powder or sterile solution, bottled for laboratory use as a cell-culture reagent by Sigma Chemical Co., St. Louis, MO; Hood and Woodart.

(ii) Cellulase and Xylanase

They are involved in the degradation plant parts made up of cellulose (a complex polysaccharide made up of repeating units of glucose). They are widely used in paper and pulp industries, in bioethanol, textiles and animal feed. Transgenic plants expressing genes for these enzymes must be protected from digestion of plant parts (autodigestion). Thus genetically engineered thermostable forms of these enzymes with high temperature optima are being used. Therefore, cellulase and xylanase, produced by transgenic plants, are inactive at normal temperatureTheir activity could be reinstated upon heating of plant extracts. Transgenic tobacco plants produced by the introduction of gene encoding catalytic domain of thermotolerant cellulose endo 1,4 α-D glucanase (E1) from *Acidothermus cellulolyticans* fused to a CaMV promoter and chloroplast targeting; show higher expression levels of protein. Recently Harrison et al., 2014 demonstrated stability of cellulase enzyme transgene expression in transgenic sugarcane and the utility of sugarcane as a biofactory crop for production of cellulases.

(iii) α-amylase

α-amylases hydrolyzes α-1,4-glycosidic linkages in amylose and amylopectin molecules of starch. It is widely used in various industries like paper, pulp, sugar, textiles, food, brewing, starch liquefaction and alcohol. A gene encoding thermostable α-amylase was isolated from *Bacillus licheniformis.* It was expressed in transgenic tobacco plants. A chimera having α-amylase gene of *Bacillus licheniformis*, signal peptide of tobacco PR-S protein (targets protein to apoplast) was transformed into tobacco protoplasts and showed much higher expression levels promising for commercial applications (Pen et al., 1992).

(iv) Aprotinin

Aprotinin is a competitive serine protease inhibitor that inhibits trypsin, chymotrypsin, kallikrein and plasmin. It is being used in medicine as an antiinflammatory and antithrombotic adjunct to cardiac surgery (Zhong et al., 1999). It was traditionally isolated from bovine lung by methods involving fractional precipitation, gel filtration, and ion exchange chromatography. Currently it is

manufactured by transient expression of the aprotinin gene in RNA (+)-strand tobacco mosaic virus vectors propagated in non-transgenic *Nicotiana* plants. This is a recombinant form of the native, bovine-sequence aprotinin, which is being marketed by Sigma Chemical Co., St. Louis, MO.

(v) Phytase

Phytase is an important supplement to livestock feed as it releases phosphates from substrates, thus ameliorates digestability and availability of nutrients. It optimizes phosphorus consumption and reduces excretion thus minimizes eutrophication. Tobacco plants have been transformed using phytate gene from *Aspergilus niger*. Also transgenic seeds of phytase producing canola have been used in livestock feed (Phytaseds^{TM}, Gist-brocades NV, Netherland) (Zhang et el.,2000)

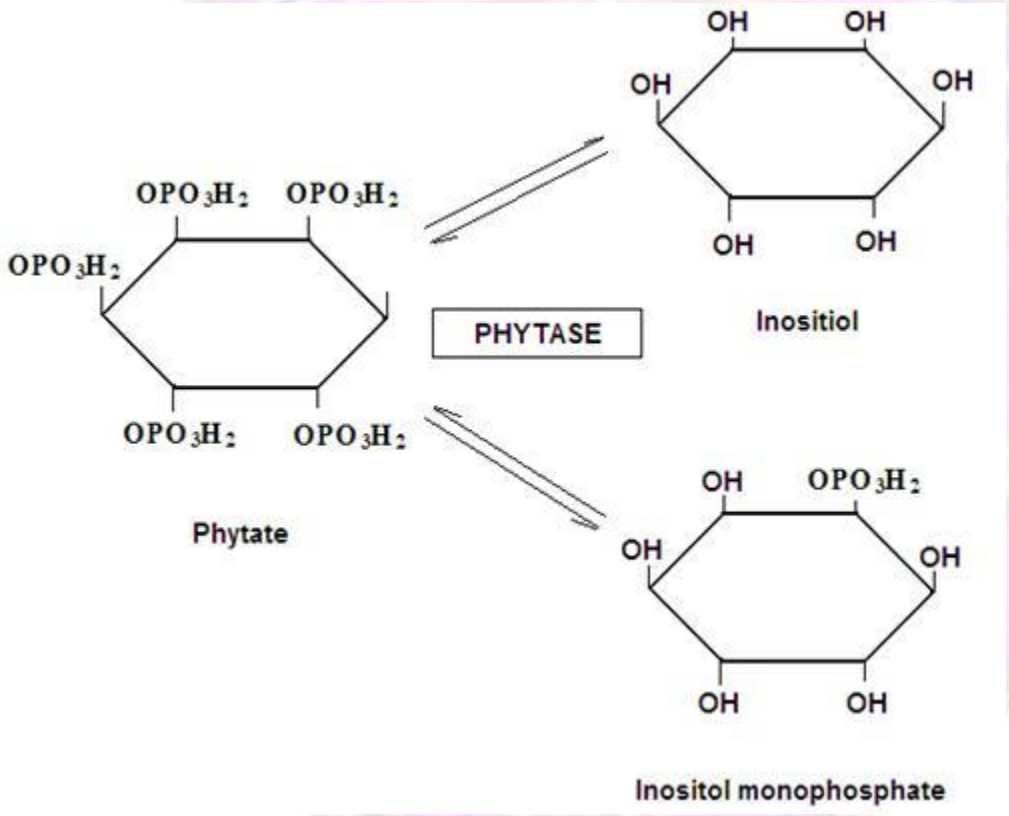

Figure: Mechanism of action of phytase.

Source: http://nptel.ac.in/courses/102103016/40(cc)

2. Bioplastics

Bioplastics are plastics derived from renewable biomass sources, such as vegetable fats and oils, corn starch, pea starch or microbes, and are generally biodegradable. They can be made up of a variety of materials including: starches, cellulose, or other

biopolymers. They are used as packaging materials, dining utensils, food packaging, and insulation.

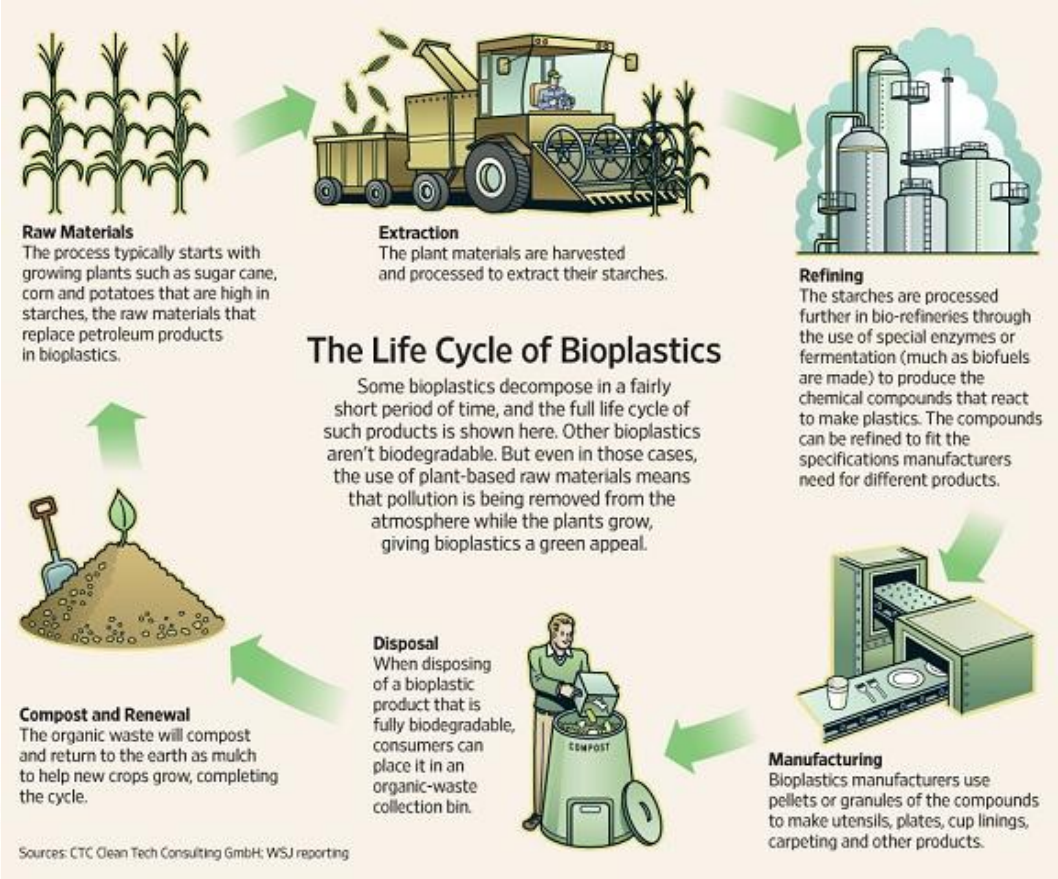

Figure: Bioplastics

Source: http://www.natural-biodegrade.com/wp-content/uploads/2013/09/life-cycle-of-bioplastics.jpg(cc)

Common biodegradable plastics or bioplastics are polyhydroxyalkanoates (PHAs) which are basically linear polyesters. They are produced by many microbes as lipid reserves and source of intracellular carbon and energy. PHAs are commercially being produced by microbial fermentation.

Various experimental studies are underway for the production of bioplastics using transgenic plants. Polyhydroxy butyrate (PHB) production is a three stage pathway involving three key enzymes : 3-Ketothiolase (phaA), Acetoacetyl-CoA reductase (phaB) and PHB synthase (phaC). Genes encoding these enzymes have been isolated from *Alcaligenes eutrophus* and cloned (Poirier et al., 1995). The cytoplasm of plant cell contains 3-Ketothiolase. Therefore only two genes (phaB and phaC) coding acetoacetyl CoA reductase and PHB synthase were transferred to develop transgenic *Arabidopsis* expressing all three of these genes. Low levels of expression were obtained.

In an another strategy, PHB was expressed in plastids where all three genes (phaA, PhaB, PhaC) of PHB synthesis was separately fused with a coding sequence of transit peptide bound to N- terminal fragment of Rubisco (ribulose 1,5- bisphosphate carboxylase oxygenase) subunit protein. They were then directed to chloroplast under CaMV 35S promoter. Firstly, transgenic *Arabidopsis* plants with each gene construct were developed. This was followed by a series of sexual crossings between the individual transformants. The transgenic plants developed yielded good quantity of bioplastics without any adverse effect on the growth and fertility of plants.

Although excellent progress has been made in recombinant hosts for the production of bioplastics, the barriers to obtaining high quantities of PHA at low cost still remain to be solved. The commercially viable production of PHA in crops, however, appears to be a realistic goal for the future. However, economically viable production of PHA in crops appears to be a realistic goal for the future (Suriyamongkol et al. 2007).

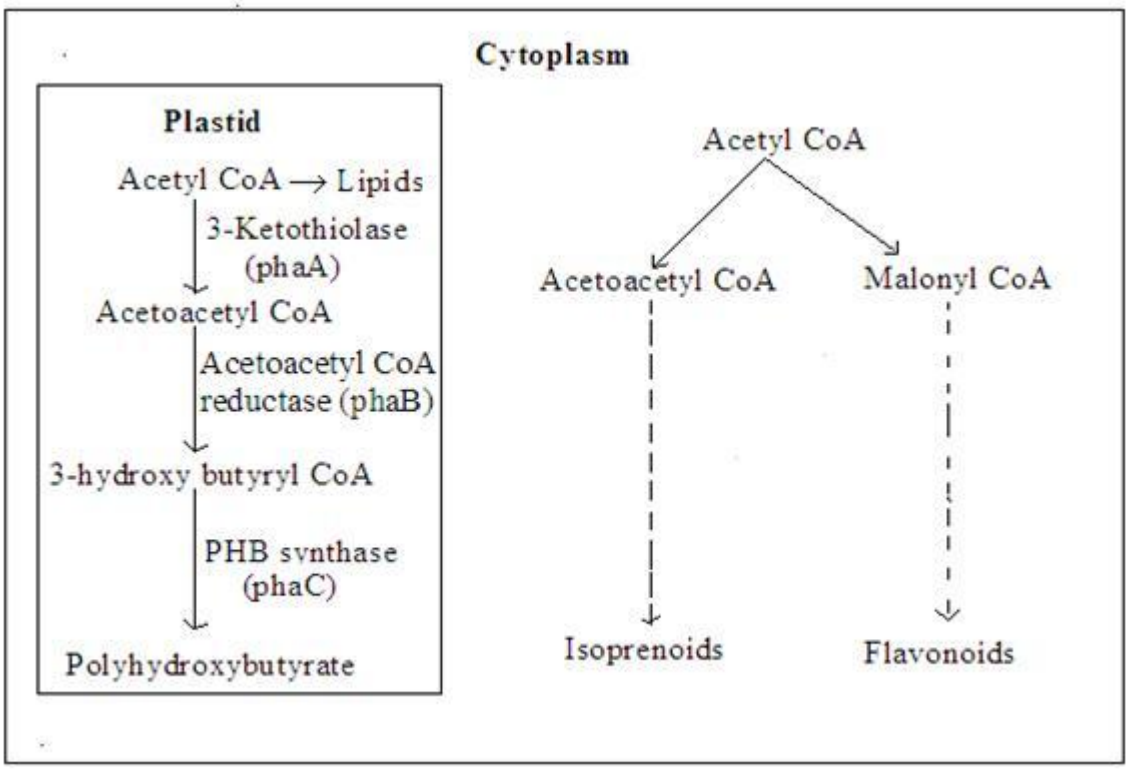

Figure: Mechanism of synthesis of PHB in chloroplasts.

Source: http://nptel.ac.in/courses/102103016/40(cc)

Production of commercially important crops

Presently, transgenic crops are being designed to introduce newer traits like resistance to various pesticides, herbicides, biotic and abiotic stresses, modification of metabolic pathways to add nutritive value.

1. Trangenic plants which are resistant to abiotic and biotic stress

The Flavr Savr™ tomato was the first genetically modified plant crop to be commercialized in 1994. These tomatoes exhibited a longer shelf life using antisense RNA technology to down regulate levels of a polygalacturonase enzyme involved in fruit ripening (Kramer and Redenbaugh, 1994).

The CRY1AA, CRY1AB and/or CRY1AC genes encoding a δ-endotoxin from *Bacillus thuringiensis* have been introduced in the Bt transgenic crops for pest control (Estruch et al., 1997; Roh et al., 2007).

In soybean, canola, cotton and maize, herbicide-resistant transgenic lines have been introduced in 1996 to simplify weed-control practices. The glyphosate-tolerant crops carry in their genome a gene derived from a strain of *Agrobacterium tumefaciens* encoding the enzyme 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase, which is insensitive to the inhibitory effect of glyphosate (Funke et al., 2006).

2. Transgenic plants with improved nutritive value

Plant proteins are the major source of dietary proteins but usually provide inadequate nutrition owing to absence of several essential amino acids. For example, Met and Cys are generally not present in legumes and Lys and Trp are missing from cereals. Genetic manipulations can be employed for improving the protein content of some crops with the aim of enhancing the essential amino acid content. Production of synthetic proteins, manipulation of protein sequences, over-expression of heterologous or native proteins, and metabolic engineering of the free essential amino acid and protein pools are some of the genetic approaches being employed. (Samuel and Qiaoquan, 2004)

In rice, a carotenoid-accumulating variety (Golden Rice) was generated by the manipulation of the provitamin A biosynthetic pathway, helping to treat vitamin A deficiencies (Beyer et al., 2002).

Figure: A Wild type rice. B Golden rice modified for the synthesis of beta carotene.

Source: http://www.goldenrice.org/

Video link for production of maize with improved nutritive quality in Haiti:

http://www.oreworld.org/qpm.htm

Link for animation of what kind of genetically modified foods would be made in future:
http://www.pbs.org/wgbh/harvest/coming/coming.html

3. Transgenic crops to yield quality oils

Plants produce oils or starch for the seedling growth. Usually up to 50% of the seed dry weight is oil. Some plants also produce oil in other parts like in fruit of olives. Basically plant oils are triglycerides and accumulate in oil bodies. They have a wide application in the foods, cosmetic and oleo chemical industry. Plant biotechnology offers potential to transfer genes that can control production of high value fatty acids into high yielding and well developed oilseed crops like canola.

First commercial product produced by changing composition of plant seed via genetic manipulations is high lauric acid canola oil. Lauric acid is present in high levels in tropical plants but not in temperate crops. Calgene, a California biotechnology company, discovered the biochemical pathway for lauric acid synthesis using California bay tree that accumulates high levels of lauric acid. Scientists cloned the gene for critical enzyme in the pathway and transformed canola. Transgenic or

genetically modified (GM) canola had dramatically increased amount of lauric acid and changed fatty acid spectrum. In 1995 first commercial production of genetically engineered quality oil was achieved using transgenic canola seeds expressing around 40-50% lauric acid.

Soyabean is the largest source of vegetable oil in the world. Most soyabean varieties produce an oil rich in polyunsturated fatty acids (PUFAs). These make the oil unstable, it gets easily oxidized and thus becomes rancid. Upon heating oil develops an undesirable color and odour. Therefore, unprocessed oil is chemically hydrogenated which add to the cost of oil and also creates trans fatty acids by conversion of cis double bond to trans. Biosynthesis of PUFAs is catalyzed by a series of enzymatic steps. The first step is the conversion of oliec acid (18:1) to linoleic acid (18:2). Gene for this enzyme was isolated and its expression was knocked down in genetically modified soyabean plants. This almost completely removed PUFAs in soyabean oil. The new genetically modified soyabean had around 85% oliec acid. This eliminated the need for hydrogenation of oil thus cutting the cost. Also due to increase in oliec acid content the amount of saturated fatty acid fell from 15% to 8%. This new oil provided greater health benefits similar to high oliec acid oils like olive oil. This GM soyabean crops were also stable in the fields. Thus high quality soyabean oil was produced and now being used commercially.

Table below list certain plant derived biomolecules:

Table: Plant derived nutritional products

S.no.	Compound	Origin of gene	Application	Plant
1.	Medium chain fatty acid	California bay tree	Food, detergent industry	Oilseed rape
2.	Mono unsaturated fatty acids	Rat	Food	tobacco
3.	Poly hydroxybutyric acid	*Alcaligenes eutrophus*	Bio plastics	Oilseed rape, *Aradiopsis*
4.	Saturated fatty acids	*Brassica rapa*	Food	Oilseed rape
5.	Amylose free Starch	*Solanum tuberosum*	Food, industry	potato
6.	Cyclodextrins	*Klebsiella pneumoniae*	Food, Pharma	Potato

7.	Fructans	*Bacillus subtilis*	Food, industry	Tobacco, potato
8.	Overexpressed Starch	*E.coli*	Food, industry	Potato
9.	Trehalose	*E.coli*	Food stablizer	Potato

Source: Author

Summary

Molecular farming is the large scale production of recombinant products in plants. It aims to harness the potential of agriculture for the production of recombinant therapeutics, industrial enzymes, quality oils, diagnostics, and bioplastics. It has the potential to produce large amounts of a desired recombinant protein. Plants have various advantages over conventional expression systems, like bacteria, yeast, mammalian cell lines and transgenic animals. Transgenic plants can be maintained, harvested and processed using normal agricultural practices and are cost effective and environment friendly. Plant biotechnology can thus be used to produce various molecules which have different commercial applications. Future holds challenges for transgenic plant derived products. Expression of these products needs to be optimized. Environmental safety and stability of products is still debatable. Regulatory considerations, ethical and legal issues still need to addressed. Nonetheless, plant biotechnology holds immense promise as a cost effective and safe industry.

Exercise

- What are the various industrial applications of plant biotechnology?
- What are plantibodies?
- List advantages and disadvantages of molecular farming?
- Plants are being used as bioreactors. Explain?
- What are quality oils and how are they produced?
- What are vaccines and how are plant derived vaccines better than conventional vaccines?

Glossary

Bioreactor: A bioreactor is a device in which a substrate of low value is used by living cells to generate products of higher value.

Biopharmaceuticals: A class of therapeutic proteins produced using genetic engineering of living organisms like transgenic plants and animals.

Bioplastics: Bioplastics are plastics derived from renewable biomass sources, such as vegetable fats and oils, corn starch, pea starch or microbiota.

Edible vaccine: Vaccines produced in transgenic plants and can be consumed orally.

Genetically modified organisms (GMOs): An organism whose genetic material has been modified using techniques of gene manipulation.

Molecular farming: The use of whole organisms, organs, tissues, cells, or cell cultures, as bio-reactors for the production of commercially valuable products via recombinant DNA techniques.

Plantibodies: Antibodies produced by transgenic plants that have been genetically moidified using an animal DNA.

Transgenic plants: Plants whose genetic material has been altered by insertion of foreign DNA using techniques of gene manipulation.

References

- Plant Biotechnology: The genetic manipulation of plants (2nd Edition); Nigel W. Scott, Mark R. Fowler, Adrian Slater; Oxford university press
- Introduction to Plant Biotechnology (3rd edition); H S Chawla; CRC Press
- Plant biotechnology ; N C Gautam; Shree publishers
- Recent advances in biotechnology; F. Mavituna; Springer Netherland
- Daniell H, Streatfield S.J.,Wycoff K., (2001) Medical molecular farming: production of antibodies, biopharmaceuticals and edible vaccines in plants; TIPS 6, 219-226.
- Awale MM, Mody SK, Dudhatra GB, Avinash Kumar, Patel HB, Modi CM, Kamani DR and Chauhan BN. (2012) Transgenic Plant Vaccine: A Breakthrough in Immunopharmacotherapeutics;J Vaccines Vaccin3:147.
- ISAAA,2007.

- Bock R. (2014). Genetic engineering of the chloroplast: novel tools and new applications. Curr Opin Biotechnol. 26:7-13.
- Hernandez-Garcia CM, Finer JJ. (2014)Identification and validation of promoters and cis-acting regulatory elements. Plant Sci.;217-218:109-19.
- Arch Virol. 1997;142(9):1857-63. Nicotiana benthamiana plants expressing beet necrotic yellow vein virus (BNYVV) coat protein-specific scFv are partially protected against the establishment of the virus in the early stages of infection and its pathogenic effects in the late stages of infection. Fecker LF1, Koenig R, Obermeier C.
- Nat Biotechnol. 2004 Jun;22(6):732-8. Fusion proteins comprising a Fusarium-specific antibody linked to antifungal peptides protect plants against a fungal pathogen. Peschen D1, Li HP, Fischer R, Kreuzaler F, Liao YC.
- Phytopathology. 1998 Dec;88(12):1367-71. Expression of engineered antibodies in plants: a possible tool for spiroplasma and phytoplasma disease control. Chen YD, Chen TA.
- Baum T.J., Hiatt A, Parrt WA, Pratt LH, Husset Rs (1996) Expression in tobacco of a functional monoclonal antibody specific to stylet secretions of root-knot nematode. Molecular plant microbe interactions,9: 382-387.
- Voss A, Nierbach et al (1995). Reduced virus infectivity in N tabacum secreting a TMV specific full size antibody. Molecular breeding1, 39-50.
- Tavladoraki P, Benvenuto E, Trinca S, De Martinis D, Cattaneo A, Galeffi P. Transgenic plants expressing a functional single-chain Fv antibody are specifically protected from virus attack. Nature. 1993 Dec 366(6454):469-72.
- Liao YC, Li HP, Zhao CS, Yao MJ, Zhang JB, Liu JL. Plantibodies: a novel strategy to create pathogen-resistant plants. Biotechnol Genet Eng Rev. 2006;23:253-71. Review.
- Weintraub JA, Hilton JF, White JM, Hoover CI, Wycoff KL, Yu L, Larrick JW, Featherstone JD. Clinical trial of a plant-derived antibody on recolonization of mutans streptococci. Caries Res. 2005 May-Jun;39(3):241-50.
- Larrick JW, Yu L, Chen J, Jaiswal S, Wycoff K. Production of antibodies in transgenic plants. Res Immunol. 1998 Jul-Aug;149(6):603-8. Review.
- Stöger E, Vaquero C, Torres E, Sack M, Nicholson L, Drossard J, Williams S, Keen D, Perrin Y, Christou P, Fischer R. Cereal crops as viable production and storage systems for pharmaceutical scFv antibodies. Plant Mol Biol. 2000 Mar;42(4):583-90.
- Zeitlin L, Olmsted SS, Moench TR, Co MS, Martinell BJ, Paradkar VM, Russell DR, Queen C, Cone RA, Whaley KJ. A humanized monoclonal antibody produced in

transgenic plants for immunoprotection of the vagina against genital herpes. Nat Biotechnol. 1998 Dec;16(13):1361-4.

- Khoudi H, Laberge S, Ferullo JM, Bazin R, Darveau A, Castonguay Y, Allard G,Lemieux R, Vézina LP. Production of a diagnostic monoclonal antibody in perennial alfalfa plants. Biotechnol Bioeng. 1999 Jul 20;64(2):135-43.
- Whaley KJ, Hiatt A, Zeitlin L. Emerging antibody products and Nicotiana manufacturing. Hum Vaccin. 2011 Mar;7(3):349-56.
- Doran PM. Foreign protein production in plant tissue cultures. Curr Opin Biotechnol. 2000 Apr;11(2):199-204. Review.
- McCormick AA. Tobacco derived cancer vaccines for non-Hodgkin's lymphoma: perspectives and progress. Hum Vaccin. 2011 Mar;7(3):305-12.
- Vaquero C, Sack M, Schuster F, Finnern R, Drossard J, Schumann D, Reimann A, Fischer R. A carcinoembryonic antigen-specific diabody produced in tobacco. FASEB J. 2002 Mar;16(3):408-10.
- McCormick AA, Kumagai MH, Hanley K, Turpen TH, Hakim I, Grill LK, Tusé D, Levy S, Levy R. Rapid production of specific vaccines for lymphoma by expression of the tumor-derived single-chain Fv epitopes in tobacco plants. Proc Natl Acad Sci U S A. 1999 Jan 19;96(2):703-8.
- Kathuria S, Sriraman R, Nath R, Sack M, Pal R, Artsaenko O, et al. Efficacy of plant-produced recombinant antibodies against HCG. Hum Reprod 2002;17(8):2054–61.
- Jain A, Saini V, Kohli DV. Edible transgenic plant vaccines for different diseases. Curr Pharm Biotechnol. 2013 Jul;14(6):594-614.
- Hefferon KL. Plant virus expression vectors set the stage as production platforms for biopharmaceutical proteins. Virology. 2012 Nov 10;433(1):1-6.
- Hood EE, Kusnadi A, Nikolov Z, Howard JA. Molecular farming of industrial proteins from transgenic maize. Adv Exp Med Biol. 1999;464:127- 47.
- Beisgen C, Hillebrand H, Herbers K.(2002) Technical enzymes produced in transgenic plants. Phytochemistry reviews. 1:79-85.
- Hood and Woodart; *Commercialization of a Protein Product fromTransgenic Maize;* Agricultural Biotechnology: Beyond Food and Energy to Health and the Environment
- Pen J, Molendijk L, Quax WJ, Sijmons PC, van Ooyen AJ, van den Elzen PJ,Rietveld K, Hoekema A. Production of active Bacillus licheniformis alpha-amylase in tobacco and its application in starch liquefaction. Biotechnology (N Y). 1992
- Harrison MD, Geijskes RJ, Lloyd R, Miles S, Palupe A, Sainz MB, Dale JL.Recombinant cellulase accumulation in the leaves of mature,

vegetativelynpropagated transgenic sugarcane. Mol Biotechnol. 2014 Sep;56(9):795-802.

- Zhong GY, Peterson D, et al. (1999) Commercial production of aprotinin in transgenic miaze seeds, Mol breed, 5 345-356.
- Zhang ZB, Kornegay ET, Radcliffe JS, Wilson JH, Veit HP. Comparison of phytase from genetically engineered Aspergillus and canola in weanling pig diets. J Anim Sci. 2000 Nov;78(11):2868-78.
- Poirier Y, Nawrath C, Somerville C. Production of polyhydroxyalkanoates, a family of biodegradable plastics and elastomers, in bacteria and plants. Biotechnology (N Y). 1995 Feb;13(2):142-50.
- Suriyamongkol P, Weselake R, Narine S, Moloney M, Shah S. Biotechnological approaches for the production of polyhydroxyalkanoates in microorganisms and plants - a review. Biotechnol Adv. 2007 Mar-Apr;25(2):148-75.
- Kramer M And Redenbaugh K (1994) Commercialization of a tomato with an antisense polygalacturonase gene: The FLAVR SAVRTM. *Euphytica,* 79, 293-297.
- Estruch Jj, Carozzi Nb, Desai N, Duck Nb, Warren Gw.And Koziel Mg (1997) Transgenic plants: an emerging approach to pest control. *Nat Biotechnol,* 15, 137-141.
- Roh J, Choi Jy, Li Ms, Jin Br And Je Yh. (2007) Bacillus thuringiensis as a specific, safe, and effective tool for insect pest control. *J Microbiol Biotechnol,* 17, 547-559.
- Funke T, Han H, Healy-Fried Ml, Fischer M And Schonbrunn E (2006) Molecular basis for the herbicide resistance of Roundup Ready crops. *Proc Natl Acad Sci U S A,* 103, 13010-13015.
- Beyer P, Al-Babili S, Ye X, Lucca P, Schaub P, Welsch R And Potrykus I (2002) Golden Rice: introducing the beta-carotene biosynthesis pathway into rice endosperm by genetic engineering to defeat vitamin A deficiency. *J Nutr,* 132, 506S-510S.
- Samuel and Qiaoquan (2004)Transgenic approaches to improve the nutritional quality of plant proteins In Vitro Cellular & Developmental Biology - Plant:40, 2, pp 155-162.

Web Links

http://scialert.net/fulltext/?doi=rjmp.2012.466.488&org=10

http://www.sciencedirect.com/science/article/pii/S0958166900002445

www.oecd.org/dataoecd/61/13/1947629.pdf

http://www1.oecd.org/publications/e-book/9301061e.pdf.

http://www3.imperial.ac.uk/manufacturingfutureslab/biotechnology

http://www.bio.org/articles/what-industrial-biotechnology