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Development of Transgenetic Organisms: Methods and Examples

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ABSTRACT: Transgenetic organisms, also known as Genetically Modified Organisms (GMO), are genetically engineered organisms which contain a foreign DNA introduced in them through biotechnology. Transgenetic organisms applied both in plants and animals. Theyare engineered using different methods/technology to produce them. The genetically modified plants involve methods such as the vector mediated method which includes Agrobacterium mediated and plant virus mediated. Additionally, direct gene transfer methods are used which include physical methods such as Electroporation, Particle Bombardment and Microinjection, along with chemical methods involving Poly ethylene Glycol (PEG) mediated transfer, Liposome fusion and Diethylaminoethyl (DEAE) dextran mediated. These techniques have been used to produce Genetically Modified plants such as Bt cotton, Golden Rice, edible vaccine, biodegradable polymers, and monoclonal antibodies. Animals, being more genetically complex, utilize other methods such as the Pronuclear Microinjection, Embryonic Stem (ES) Cell transfer, Viral vectors, Somatic Cell Nuclear Transfer (SCNT), Primordial Germ Cells (PGC) Mediated Gene Transfer and Sperm Mediated Gene Transfer. Genetically modified animals have been used to improve xenotransplantation, nutritional value of milk, yield of wool from sheep and produce monoclonal antibodies along with artificial spider silk. The development of such GMO's for the field of pharmacy are regulated by the Central Drug Standard Control Organization (CDSCO) and Department of Biotechnology (DBT). The GMOs overall are regulated by Sections under the Environment Protection Act (EPA),1986 and Rules 1989.

Keywords: Genetically Modified Organism, Agrobacterium, Pronuclear Microinjection, Regulations

I. INTRODUCTION:

Transgenetic organisms are genetically engineered organisms which contain a foreign DNA introduced in them through biotechnology.The genetic engineering technology aims to produce a technique for the rapid integration of a 'new' or modified gene into an unrelated species to enhance or supress a certain trait. The idea of creatingtransgenetic organisms was to improve the field of medicine by introducing newer techniques and drugs to improve the treatment options and longevity of the patient. Additionally, it has created a huge impact in the field of agriculture and animal husbandry by enhancing the crop yield. This was brought about by introducing pest resistant plants and enhanced yield of meat and milk from animals. The nutritional value of milk, meat and vegetables has also been enhanced. GMO products have also been used widely in other fields to improve the commercialization of certain products. The first transgenetic organism was created in 1974 when Annie Chang and Stanley Cohen expressed Staphylococcus aureus genes in Escherichia coli.Ever since then,advancements in this field have been by transforming more complex animals such as Dolly the sheep and Rosie the cow. Various techniques have been used to produce these transgenetic organisms such as the vector mediated method and the direct gene transfer technique in plants and animal. The Agrobacteriumvectorhas been a popular method to transform the plants along with the plant virus vectors and retroviral vectors. The direct gene transfer methods include electroporation and biolistic, commonly used in in plants, whereas pronuclear Microinjection, Somatic Cell Nuclear Transfer(SCNT)are popularly used in animals. Due to the increase in research in the field of transgenics along with the demand of GMO products, the debate with respect to the ethical ground of transgenic organisms has constantly been brought about. These debate lead to the need of the regulation of GMO's done by the Ministry of Environment, Forest, and Climate Change (MoEFCC) and organizations such as Central Drug Standard Control Organization (CDSCO), under the Ministry of Family and Health Welfare

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(MoFHW) and Department of Biotechnology (DBT), under the Ministry of Science and Technology have laid the guidelines. Guidelines are laid under sections mentioned in the Environment Protection Act, 1986, Drug and Cosmetic Act, 1945 and the Rules of 1989 under Drugs and Cosmetic Act,1945.

II. TRANSGENETIC PLANTS:

Development of the first transgenic plant was when the tobacco plant was inserted with a gene responsible for bacterial antibiotic resistance 'nptII'. Afterward, various transgenetic plants were developed which possessed important agronomic traits including pest resistance and drought resistance. The process of transgenesis incorporated both the monocots and dicots which might be amenable to genetic modification.

A)VECTOR MEDIATED: Vector acts a vehicle which aids in transportation of the desired gene into the target host for replication and expression. It consists of an origin of replication, multicloning or recombinant site and selectable marker. The origin of replication is responsible for the initiation of the vector replication, which unwinds the vector by binding to a protein complex and replicates with the help of polymerases. The gene of interest can be placed inside after splicing of the required restriction sites, which are multiple unique sequences present in the multicloning site, using restriction enzymes. It is responsible for the plasmid recombination which is site specific. The selectable markers are responsible for the validation of the gene insertion. The most common vector used for development of transgenetic plants includes Agrobacteriumvector and Plant virus vectors.

AGROBACTERIUM METHOD: Agrobacterium is a soil bacterium that is gram negative and rod shaped. Two species of the Agrobacterium, namely Agrobacterium tumifaciens and Agrobacterium rhizogenes are used for the plasmid vectors. A.tumifaciens introduces the gene in the host through crown gall disease or tumour, which means tumorigenesis in the neck of root. A.rhizogenesfollows the mechanism of hairy root disease, due to which the formation of proliferative multibranched adventitious root occurs in order to incorporate the new gene. As a genus, Agrobacterium can transfer DNA known as T-DNA (transferred DNA) into the DNA of a wide range of organisms which include dicot and monocot angiosperms along with gymnosperms.

The process of a stable Agrobacterium transformationfollows the steps as given below.

i. The recognition of the chemical signal of host is done
ii. The vir gene is activated in the Agrobacterium
iii. The Agrobacterium is attached to the plant cell
iv. The virulence protein is activated and transported
v. T-DNA strand is produced
vi. The virulence protein and the T-DNA is transferred into plant nuclear
vii. The T-DNA integration into plant genome

Ti PLASMID

Ti plasmid is introduced into the plant through the A.tumifaciens species. Ti plasmids are in the range of 200-800kbp. The three prime area of importance in the Ti plasmid are the T-DNA, the virulence region (vir) and opine catabolism region.The T-DNA is a border sequence which means it is imperfect repeats of conserved 25 base pair on the T region. The virregion consists of seven major loci and is responsible for encoding the bacterial protein machinery which process and transfer the T-DNA. The activation of the required genes which elicit response on the Ti plasmid is done by the virA and vir G proteins. The virB, virC, virD, virE and virFmediate the processing, transfer and integration of the T-DNA into the plant cell. Opines are secondary metabolites coded by the Ti plasmids through the Opine catabolism region which are derived from amino acids and sugars. Ti plasmids are also responsible for the synthesis of phytohormones- auxin and cytokine. These three oncogenes together are responsible for the formation of the tumour in plants which leads to the crown gall disease. The uncontrolled proliferation of plant cells for tumour formation is another function of the phytohormones. Opines on the other hand act as the carbon source for the A.tumifaciens plant. During the genetic transformation, the reporter gene along with the gene of interest is integrated in place of the tumour inducing genes, and is known as disarmed Ti plasmid.

The large size of the Ti plasmid posed certain limitations which lead to formation of the co-integrative vector system and the binary vector. The co-integrative system utilizes an intermediate vector, most commonly E.coliin addition to the disarmed Ti plasmid. The homologous

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recombination of these is then reintroduced into the Agrobacterium. Finally, this is incorporated into the plant host. On the other hand, binary vector is a two plasmid system which involves a mini vector and a helper vector. The mini vector is an integration of the E.coli and A.tumifaciens consisting of the Ti plasmid whereas the helper vector is a wild Ti plasmid which is devoid of the T-DNA. It assists by producing a template for the genes involved in the gene integration and transfer.

Ri PLASMID

Ri plasmid is introduced in the plant through the Agrobacterium rhizogenes species. Ri plasmids consist of rol genes and orf genes. Among these genes,rolA(orf10), rolB(orf 11), rolC(orf12), rol D(orf15), orf8, orf13 and orf14 are mainly responsible for the hairy root formation. The T-DNA also consist of mas, aux and ags genes which encodes opines and auxin. The major role of Ri plasmids is to produce secondary metabolites of plants through transgenesis. Amount of secondary metabolites derived from plants naturally is very minimal and cannot sustain the requirement of these substances in the pharmaceutical industry. Inorder to enhance their production efficiently and quickly, the technique of genetic modification usingRi plasmid was discovered. The secondary metabolites in plant are formed utilizing various metabolic pathways derived from the primary metabolic pathways. These secondary metabolites are used as excipient and most importantly as active components in drugs for treatment of a variety of ailments. Some of the secondary metabolites used as the active pharmaceutical ingredient for ailment of certain diseases are enlisted below in Table 1.

DRUG NAME	COMPOUND CLASS	USE
ATROPINE	Tropane Alkaloid	Anticholinergic
CODEINE	Phenantrene Alkaloid	Analgesic, Antitussive
QUININE	Quinoline Alkaloids	Anti-malarial
QUINIDINE	Quinoline Alkaloid	Cardiac Depressant
DIGOXIN	Steroidal Glycoside	Cardiac Simulant
PICROTOXIN	Bitter Glycoside	CNS Stimulant
VINCRISTINE	Indole Alkaloid	Antineoplastic

TABLE 1: Secondary metabolites, their class and use

Ri plasmids have been used extensively owing to their advantagesof greater production which can be contributed to the following factors:

i. Hairy roots proliferate rapidly and do not require use of phytohormones for the enhancement of the production.
ii. There is a high yield for specific metabolites especially for those produced in the roots of the plant naturally. For those metabolites produced in green part of the plant, yield can be increased using modified hair root technique.

The use of Ri plasmid technique has extended to all the categories of secondary metabolites which include alkaloids, glycosides, flavonoids etc. The increase in yield of glycosides and alkaloid content using A.rhizogenes has been exhibited through the example shown below of picrotoxin and quinoline, respectively.

Picrotoxin is a Bitter Glycoside is derived mainly from PicrorhizakurroaRoyle(Plantaginacea). These have a broad medical application which include CNS stimulant, antidote for barbiturate poisoning and even antiviral effects. In a study by Janhvi Mishra et al, 2010 it was found that the hairy root culture enhanced picrotin from less than 1µg/g

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to8.8µg/g dry weight and picrotoxinin level from 10µg/g to 47.1µg/g dry weight.

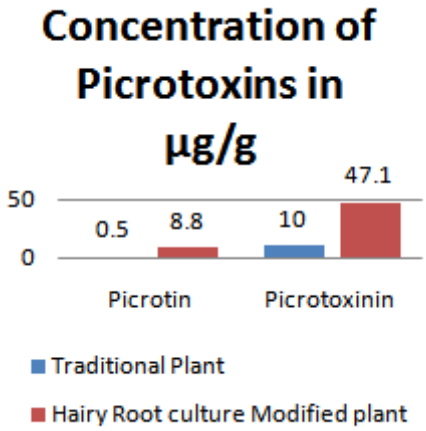

FIG 1: The quantity of secondary metabolitesproduced in Piccorhiza normally and using Ri plasmid.

PLANT VIRUS VECTOR: Viruses are submicroscopic intracellular obligate parasites that replicate inside living cell of organism by utilizing the host machinery. The viruses used for plant transformation are genetically modified and are used as an alternate source for the genetic modifications of plants. They possess efficient machinery and comprehensive genome helping them to be utilized as plant vectors. Some of the common viruses employed for this purpose include Cauliflower mosaic virus (CaMV), Tobacco mosaic virus (TMV), Alfalfa mosaic virus (AMV), Potato virus X (PVX), and Cowpea mosaic virus (CPMV). This is also utilized in both monocot and dicot plants. The Wheat streak mosaic virus (WSMV) and Barley stripe mosaic(BSMV) are commonly

Quinoline alkaloid is derived from plants belonging to various families including Ranunculales, Papaveraceae, Geraniales, Rubiacea, Solanales, and Convolvulacea. Quinolines are most commonly used as antiviral and fungicidal. In an experiment by Hamil et al in 1989, it was concluded that the concentration of quinoline in Cinchona officianalis 'Ledgeriana' increased from less than 1µg/g to up to 50µg/g. Quinolines present in cinchona include Quinine, Quinidine, Cinchonine and Cinchonidine.

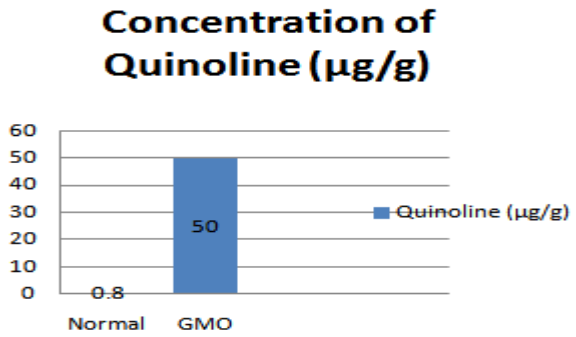

FIG 2.The quantity of secondary metabolites produced in Quinoline normally and using Ri plasmid employed in monocots whereas the TMV has its use in dicots

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These viruses are used in genetic modification of plants through two methods. The first method involves using virulence of virus by incorporating the gene of interest in the wild type virus which is capable of infecting the plant. The second approach involves the formation of 'deconstructed' viruses in which the undesired viral gene is replaced with the gene of interest such as the antibiotic resistance gene.

B) DIRECT GENE TRANSFERS: Direct Gene Transfer is the method of direct incorporation of the foreign DNA into the plant nucleus. It is a less common method used in the genetic transformation of plants. These include a wide array of physical and chemical methods which aid in the disruption of the outer mebrane of the plant cell . This allows the entry of the exogenous DNA into
the plant cell. Physical methods utilize mechanical means to achieve the disruption of the cell mebrane. These include Particle Bombardment, Microinjection, Electroporation and Silicon Carbide fibers. Particle bombardment or biolistics, also known as microprojectile, is the most common physical method employed in the field of Genetic Modification. In this method gold or tungsten particle is used to coat the DNA particle which is then shot into the plant cell under high pressure using a gene gun. The penetration of the coated DNA occurs due to its rapid movement through the thick plant cell wall. This then directs the DNA into the nucleus of the plant cell. The bombarded DNA then incorporates into the chromosome of the plant cell after disintegrating itself from the metal particles. Although it can be used in a wide variety of monoct and dicot plants, its use becomes limited owing to the need of the special instruments. Additionally, there is always a chance of the foreign DNA being bombarded ino the cell organelles instead of the plant nucleus which ultimately leads to failure of the process. Electroporation, is another physical method utilized for the transformation of cell. During this process electric impulses facilitate the entry of the foreign gene. The electrical impulses are responsible for production of pores on the cell membrane of plant cell which is placed in a buffered solution. These transient pores allow entry of the foreign DNA into the plant cell. However, this method is confined to plants with protoplasts and hence is not used widely. Chemical Methods widely used in gene transfer as they cause disruption of the cell membrane using chemicals. This includes techniques such as Liposome fusion, Poly Ethylene Glycol(PEG) mediated transfer and Diethylaminoethyl (DEAE) dextran mediated tranfer. PEG destatbalizes the cell mebrane with the help of divalent cations which aids in incorporation of the foreign gene by increasing permeability. Liposomes deliver the foreign gene particle by entrapment of the DNA and acting as a vehicle for its transfer. Due to its lipophillic nature it easily travels across the protoplasts. However, these methods have very limited use in plant genetic modification as they can only be utilized in plants with protoplasts.

Table 2 summarizes the transformation techniques used in genetic modification ofplants.

METHOD	FEATURES
VECTOR MEDIATED	
Agrobacterium Method 1. Agrobacterium tumifaciens 2. Agrobacterium rhizogenes	Agrobacterium is applicable to a wide variety of plants and is the most common method. 1. A.tumifaciens utilize Ti plasmids to produce a crown gall disease and it is used to produce pest resitant and draught resistant plants. 2. A.rhizogenes use Ri plasmid to produce hairy root growths and is used for production of secondary metabolites.

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Plant Virus Vector	It expresses high amount of trangenes and is efficient
DIRECT GENE TRANSFER	
Physical Methods	
Electroporation	Utilize electrical impulse for gene transfer. Limited to protoplasts.
Microinjection	Delivers DNA directly through microinjection. Only transforms one cell at a time and requires highly trained personnel.
Particle Bombardment	Use of biolstic gun to deliver coated DNA particle into host. Highly expensive process.
Silicon Carbide fibers	The cells created by suspension must be regenerable
Chemical Methods	
Polyethylene glycol (PEG) mediated	Increases the permeability of protoplasts allowing entry of the foreign gene into plant. Limited to protoplasts.
Liposome fusion	Acts as a vehicle for DNA transfer. Limited to protoplasts.
Diethylaminoethyl (DEAE) dextran mediated	Limited to protoplasts

C)APPLICATIONOF GENETICALLY MODIFIED PLANTS:

Bt Cotton

Bt cotton is one of the genetically modified plant that has been widely accepted. It is being used in the Indian market since its approval by the Genetic Engineering Approval committee (GEAC) of Government of India in 2002. The abbreviation Bt stands for Bacillus thurengenesis which is the microrganism utilized for genetic modification of the plant. This incorporates endospore of Bt toxin into the plants to induce pest resistance against some insects. The ingetion of these toxins by the pest results in its death. The first Bt crop was Bt tobacco produced in 1985 by the Plant Genetic System, Belgium. In 1995 the Environment Protection Agency in USA approved the commercializations of the Bt crops. The major pest which led to huge economic losses in cotton (upto 80%) was the bollworm. There are three types of bollworm which include American bollworm (Helicoverpa armigera), Pink bollworm (Pectionphora gossypiella) and Spotted bollworm (Earias vitella) out of which Helicoverpa has been attributed as the major crop yield reducing pest. Before the development of Bt technology pesticides were employed to battle this problem. However, problems such as pest resistance, secondary pest resurgence, and environmental contamination due to use of these pesticides excessively emerged. Hence, Bt plants were produced and cultivated. The production of Bt cotton is done using the following steps:

i. Identification of effective gene (Cry1Ac gene& Cry1Ab gene)
ii. Agrobacterium mediated transfer of the gene into cotton
iii. Regeneration of the modified plants from protoplasts, tissues or callus
iv. Enhance production level of the plant
v. Ensure proper integration of genes to carry forward the plant through normal reproduction

This genetic modification helped enhance the global cotton industry in social, economic, and environmental aspects. It has imroved crop management effectiveness, reduced utilization of pesticide indiscriminately, improve yield of crops, increased profitability of cultivating these plants

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and allow the agricultural oppurtunity of growing cotton in areas of severe pest infestation. This further benifitted the environment by reducing pesticide run off, reduction of air and soil pollution due to insecticide use.

GOLDEN RICE

Golden Rice is a biofrotified plant which was introduced in the year 2001 by International Rice Research Institute (IRRI) in the Philippines. Biofrotification is the process of increasing nutritional value of crops. The project was funded by the Rockefeller Foundation after it was proposed to them by two professors, Ingo Potrykus and Peter Beyer in 1999. Studies and surveys showed a deficiency of vitamin A in the people residing in developing countries to be prevalent. Most developing countries have a carbohydrate rich diet with rice as their staple food and this lacked vitamin A leading to its deficiency. This deficiency of vitamin A leads to dryness of eye, which if left untreated progresses to blindness, reeduces immune responses, and cause an overall increase in the severity and mortality of infections. Hence, rice was genetically modified to produce beta-carotene which is the precursor of vitamin A. The biofortification of the rice plant was done using two genes,a plant phytoene synthase (psy) and a bacterial phytoene desaturase (CRTI). The psy gene utilizes endogenously synthesized geranylgeranyl-diphosphate (GGPP) to form a phytoene which is a colourless carotene.The crtIgene encodesa bacterial carotene desaturase that incorporate conjugation by the addition of four double bonds. This allows the accumulation of beta-carotene in the grains which was normally seen in the green parts of the plant. Both the genes together lead to the formation of α and β carotenes along with variable amount of oxygenated carotenoids. A study by Guangwen Tang et al, 2009 showed that golden rice produced upto 35µg β-carotene per gram of rice which was completely absent in normal rice.

Edible Vaccines

Vaccines stimulate our immune system by y tricking the body to believe that it has been exposed to the antigen, for which the antibody has been produced. Similary edible vaccines trigger the immune response and helps to combat certain diseases like the normal vaccines. These are produced by the genetic modification of a plant and an animal based substance. The concept of Edible Vaccines was brought by Dr. Arntzen in the year 1990. It envisions to deliver the subunit of vaccine by incorporating them into edible fruits and vegetables. The steppingstone for the formation of edible vaccine was when Maison et al successfully expressed surface antigen of Hepatitis B in tobacco plant. They further expnaded this research to express Hepatitis B and heat labile toxin B in potato and tomato plants. The traditional vacciness exhibit certain limitation such as the high cost, specific storage temperature, need of an effective cold chain management, and the method of adminstration being painful. Hence, the edible vaccines are produced using genetic engineering. The edible vaccines primarly stimulate mucosal immunityalong with some effect on the systemic immunity.It triggers a specific response to T and B cell which also generate memory cell for the subsequent attack by the antigen. In the intestine the major route for the capture of antigens is through the Microfold(M) cells. These capture the antigens from lumen and move to Antigen presenting cells in the peyers patches. Of these APC's, Dendritic cells are the most powerful trigger for an adaptive immune reaction.

The production of Edible vaccines requiresgenetic engineeringmechanism in which the foreign gene is incorporated into the plant by using a vector such as Agrobacterium or a plant virus like TMV. Sometimes, the direct gene transfer method is used of the most common method is biolistic resulting in chloroplast transformation.

Table 3depicts the plants used in manufacturing different vaccines for various diseases along with their description.

Although Edible vaccines were approved by the National Institute of Allergy and Infectious Diseases in 1998, USFood and Drug Adminstration (USFDA) has not approved any edible vaccines yet. There is still enormous scope and potential in the research of these vaccines.

BIODEGRADABLE BIOPOLYMERS

Rampant use of plastic has been a major environmental issue since long. Environmentalists constantly emphasise on the need of biodegradable alternatives. Although paper bags and jutes have been employed, they failed to completely eliminate the use of plastic due to their various limitations. Hence, Gentic modificationin certain plants was used to produce biodegaradble bioplymers. One such example is the Polyhydroxyalkanoates (PHA's), a biodegradable natural polymer synthesized in many microorganism, which is produced in plants using genetic engineering.

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Polyhydroxyalkanoates accumulate in cytoplasm of the bacteria and is used as intracellular energy along with carbon reserves for them. PHA's appear as round shape granules, are of the size 0.1-0.2µm and have 600-3500 identical monomers. Earlier the extraction of these PHA's was done using bioreactors, but this posed difficulty due to its unprofatibility. The process required nutrient substrates of high cost. Therefore PHA's were transgenetically produced in plants. The first successful plant in which PHA's were produced was Arabidopsis thalliana in the 1900's. This has since then been used as model plant for the synthesis of PHA's.

Acetyl Co-A is the main substrate in plants that are essential for the synthesis of PHA's. Hence, cellular structures containing Acetyl CoA, such as cytoplasm, peroxisome and plastids were targeted for the formation of PHA's. The transgenetic plants were created by incorporating the three genes phaA, phaB and phaC using a vector into the desired host. Poly Hydroxy Butyrate is the most common PHA produced. These are widely used as a substitute for plastic and also offers its assistance in the field of animal husbandry, crop cultivation, medicinal implants, and research. Poly hydroxybutyrate and PHBV [Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)] acts as scaffolding to regenerating cells of the blood vessels and Gastro intestinal tract as shown by a study conducted in rats, dogs and sheep. In the field of pharmaceutics, PHA's are a promising candidate for the development of anti-HIV drugs, anti-cancer drugs, antibiotics etc. Figure 3shows the percentage of PHB produced in plants using genetic engineering.

Table 3: Examples of Edible Vaccines, their use, advantages and disadvantages

PLANT	VACCINE	DESCRIPTION (Advantage/Disavantage)
Potato	Tetanus, Diphtheria, Hepatitis B, Norwalk virus, Mink enteriris virus, E.coli enteritis, Rabbit Haemorrhagic virus	• Potato offers ease of transformation and propagation. • Cooking leads to denaturation of antigen and hence use is limited
Rice	Hepatitis B surface Antigen (HBsAg)	• Commonly used in baby food and has high expression of antigen • Grows slowly and requires glasshouse condition
Banana	Hepatitis B surface Antigen (HBsAg)	• Does not need to be cooked and hence no denaturation • However takes 2-3 tears to mature and spoils fast after ripening
Tomato	SARS virus, Norwalk virus, CT-B protein(*Vibrio choleras* B toxin), HBsAg. Pneumonia, Septicaemia, Bubonic plague	• Grows quickly • ontains vitamin A which boosts immune system • Spoils easily
Lettuce	E.coli enteritis, Glycoprotein E2(swine fear hog pest virus), HBsAg	• Can be used in raw form • Utmost effective plant
Tobacco	Norwalk virus, VP1 protein (chicken infectious anaemia). Hepatitis B	• It is not an edible plant • Used as a model for edible plant vaccine
Alfa Alfa	Glycoprotein E2 (Hog pest virus)	• This vaccine was mainly used for veterinary purposes

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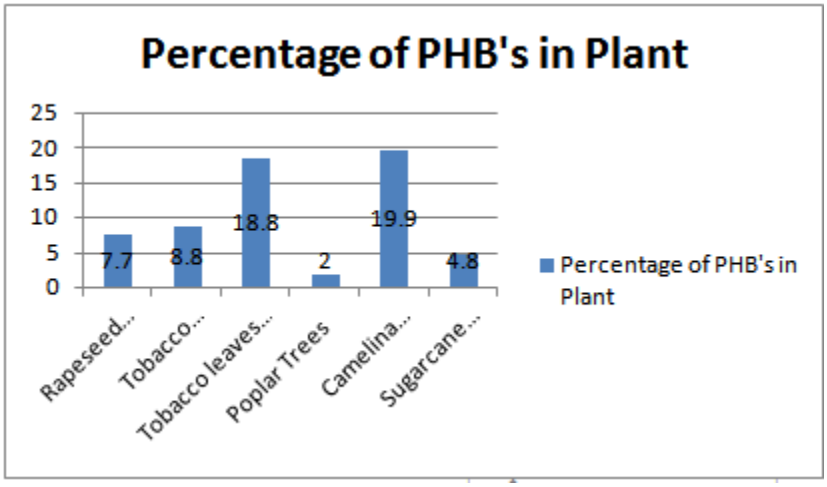

FIG 3: Percentage of PHB produced in plants using genetic engineering.

Mammalian Antibodies

In recent years, monoclonal antibodies are extensively used in the field of medicine to treat various diseases. Hence, the production of these antibodies has to be done in a large scale in order to meet with their growing needs. For this purpose techniques such as genetic engineering are utilized in the production of the monoclonal antibodies. The first successful transformation of a plant producing mammalian antibodies was seen in 1989 in a tobacco plant. Inorder to produce mammalian antibodies the tobacco plant is transformed using Agrobacterium vector which instils the code for the complementary DNA (cDNA) of the sequences of the Heavy chain (HC) gene and the Light chain (LC) gene. Additionally, a short DNA sequence is combined which is used as a linker to the HC and the LC. The mammalian antibodies are first confirmed in the plant by using Polymerase Chain Reaction (PCR) technique. Once confirmed the antibodies from the plant are then isolated using extraction buffer, purified using techniques such as centrifugation, analysed using SDS-PAGE technique and quantified by ELISA. The monoclonal antibodies from plants represent the fastest growing class of biopharmaceuticals. They have shown an advantage over the mammalian cultured monoclonal antibodies by being cost effective, utilizing less expensive machinery, possessing high scalability and increased safety. They are extensively used in the pharmaceutical industry for the treatment of various autoimmune disorders, cancer and viral infections.

D)TRANSGENETIC ANIMALS: Similar to the plants, various experiments were performed to transgenetically modify animals. The first transgenetic modification in animals was reported in 1974 where a mice was injected with SV40 virus in the early stage of mice embryos by the virologist Rudolph Jaenisch. A continuation of this was done at the Salk Institute followed by Fox Chase Cancer center by the mouse embryologist Beatrice Mintz.The transgenetic modification in animals can be carried using various methods. Presently, transgenetically modified animals are employed in various fields such as Agriculture, medical, textile industry etc. The various methods to develop transgenetic animals are described below in Table 4.

TECHNIQUES	DESCRIPTION
PRONUCLEAR MICROINJECTION	Applicable to wide range of species Transferred using pronucleus of either male or female
EMBRYONIC STEM CELL (ESC) MEDIATED GENE TRANSFER	Transfer utilizes mice blastocyst
VIRAL VECTOR	Results in chimeric animals
SOMATIC CELL NUCLEAR TRANSFER (SCNT)	Used to produce cloned animals
PRIMORDIAL GERM CELL (PGC) MEDIATED	Used for avaian species

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TRANSFER	
SPERM MEDIATED TRANSGENESIS	Modification of pronuclear microinjection using sperm cells

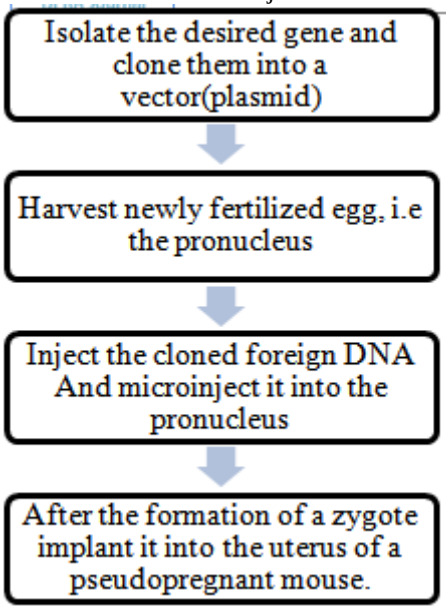
TABLE 4: Methods to develop Transgenetic animals

PRONUCLEAR MICROINJECTION

Pronucleus is a haploid nucleus which is a sperm cell that has entered the ovum, but has not fused with the genetic material of the ovum. During this process the foreign DNA is microinjected into either the male or female Pronucleus. Gordon and colleagues were the first to describe the process of pronuclear microinjection. However, the success rate of this method to incorporate the foreign genome is only 0.5-3%in which rats have 3% whereas rabbits have less than 1%.The process of pronuclear injection is as shown in Figure 4.

Since the process is a randomized one, the expression of the foreign gene may or may not be expressed in the offspring. This arises when the DNA incorporates itself into the section of host DNA which is does not express itself. Along with this, the tediousness of the process and limitation on the number of cells that can be handled at a single time, this process is not used commonly for animal transgenetic organisms.

FIG 4: Pronuclear Microinjection methodology



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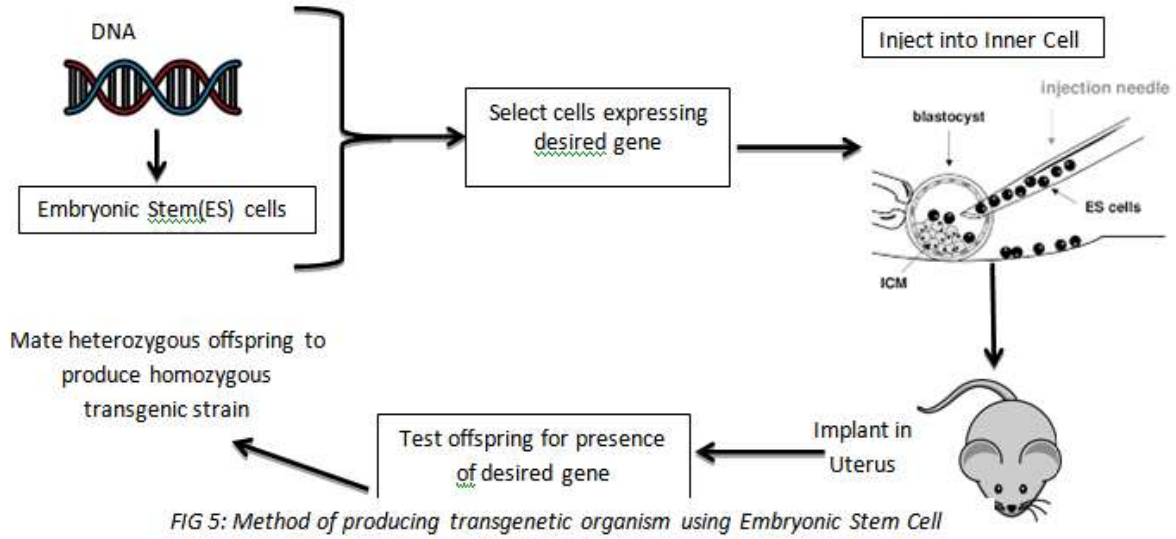

FIG 5: Method of producing transgenetic organism using Embryonic Stem Cell Transfer Method

EMBRYONIC STEMCELL MEDIATED GENE TRANSFER

Embryonic Stem (ES) cells are cells capable of differentiating to any body cell type, i.e they are totipotent in nature and are derived from the early stages of embryo. ES cells are harvested from the inner cell mass (ICM) of mice blastocysts and can be grown in culture. The desired host DNA is prepared by recombinant technology and placed in a culture medium. The ES cells are then transferred into the host DNA while it is still in the culture medium. The successfully transformed cells are then placed in the ICM. A successful transgenic strain is established, once the embryos in the uterus after the implantation of the blastocysts in a pseudo pregnant mouse are extracted and tested. The diagrammatic representation of the method is shown in Figure 5.

Although this method was successful in mouse, it was difficult to continue in the more complex organism such as mammals. In these animals it also involves a prolonged time of generation and high cost of maintenance of the multiple chimeric animals for the germ line transmission test.

VIRAL VECTOR

Similar to plants, viral vectors ay also be used for producing genetically modified animals. The viral vector most commonly employed to produce transgenetic animal includes Reterovirus. It utilizes the process of retroviral tranduction of the male germ line cells. The virulent gene of the virus is replaced by the desired foreign DNA to create the trans DNA. Finally the process of transfection (gene therapy) is used to transfer the trangene into the host animal. Since the genetic material of a reterovirus is a RNA, the incorporation of this into the host animal cell results in the formation of a chimera, i.e the organism is composed of cells with more than one distinct genotype. This is then inbred for atleast 20 generations until trangenic homozygous offspring are born. Viral vectors offer various advantages to the process of gene transfer such as being able to successfully incorporate a new gene in a wide species of animals, simplicity of the procedure and a relatively high titer of recombinant reterovirus. However, the use of this method is limited as they may cause mutagenesis due to random integration into the chromosome. It also exhibits poor invitro in vivo correlation as it requires cell divison for integration.

SOMATIC CELL NUCLEAR TRANSFER

Somatic Cell Nuclear Transfer (SCNT) is the technique of incorporating the nucleus of somatic cell into the cytoplasm of the desired host organism. In this process the somatic cell nucleus is incorporated into enucleated oocytes which are at metaphase-II stage of the cell cycle to produce identical offspring. This method is known as cloning. SCNT involves critical steps including the donor cell type, invitro culture condition of donor cell, activation of oocytes and the stage of cell

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cycle for recipient oocytes. It has been used successfully to create several animals including the first cloned mammal, Dolly the sheep in 1996. The nucleus of a mammary gland cell of a Finn Dorset ewe was fused into the enucleated embryo cell taken from a Scottish Blackface Ewe. As a result, Dolly was the clone of the Fin Dorset ewe. The major advantage of SCNT is that it allows pedetermination of sex, and phenotype of the produced trangenetic organism and hence can be used to enhance certain traits such as the amount of milk produced resulting in its mass production. The success rate of SCNT lies between 1-3% as it is less efficient and has a high mortality rate for the foetus and embryos.

PRIMORDIAL GERM CELL MEDIATED GENE TRANSFER

Primordial Germ Cell (PGC) mediated gene transfer is a method of transgenesis commonly used in avian species, as they have a gene sequencing different from that of mammals. Primordial germ cells are the precursor of the oocytes and spermatozoa in the form of primary undifferentiated stem cells. This technique is preferred in the avian species over the ESC mediated gene transfer as it is diffcult to introduce foreign DNA into zygote of birds as they show discoidal meroblastic clevage and have large amount of yolk.The process of PGC mediated transfer is as shown in figure 6.

This method is used to produce gentically engineered chickens for a higher production of meat as it is an important source of protein worldwide. In the field of Phamaceutics, chicken eggs are being transgenetically modified to produce a drug using this technique.

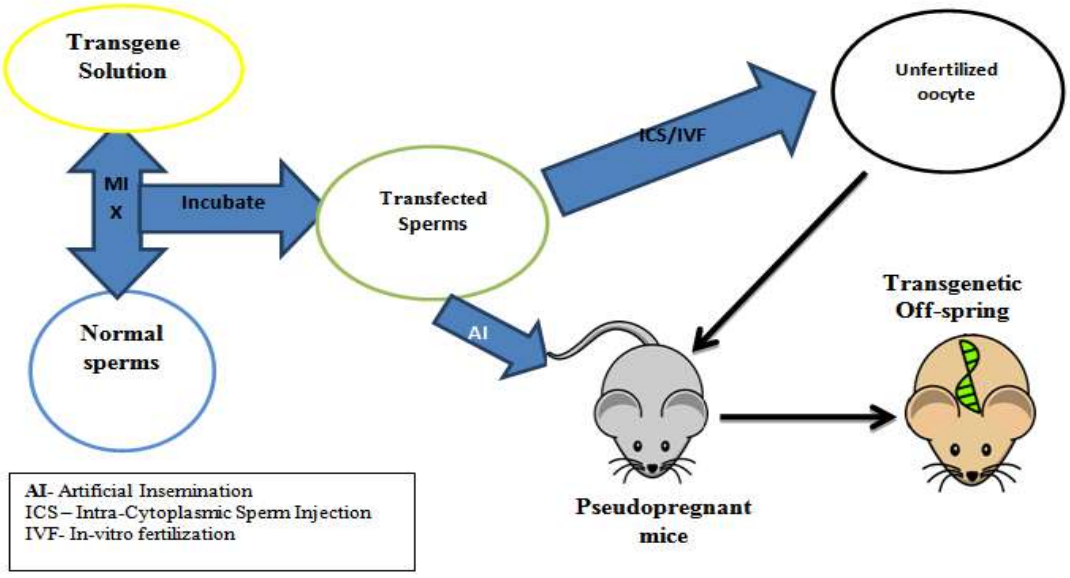

FIG 7: Diagrammatic Representation of Sperm Mediated Genetic Transfer

SPERM MEDIATED TRANSGENESIS

Sperm cells being utilized as vectors for transgenesis is an alternative method to pronuclear microinjection. Sperm cells have the capacity to inculcate foreign DNA into the oocyte during the process of fertilization and produce transgenic organisms. The fertile donor testis are cultured from a single cell susupension. This cell is then microinjected into the lumen of seminiferous tubule of an infertile mice. The sperm carrying the transgene is then transferred to the oocyte using Intra Cytoplasmic Sperm Injection (ICSI) or In-Vitro Fertilization (IVF). The diagrammatic representation of the method is as shown in Figure 7.

APPLICATIONS OF GENETICALLY MODIFIED ANIMALS

Xenotransplantation

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The transfer of living cell, tissues, or organs from one specie to another is known as Xenotransplantation. Throughout history, porcine tissues/organs have been used in xenotransplantation due to reduced availability of human donors and high cases of required transplantation. Additionaly, they have a higher acceptability in human host as compare to other animals. However, the rate of rejection of these organs by the body was high and only 45% of hosts receiveing porcine organs survived according to a data by the CDC. Rejection of these organs may present itself as hyperacute rejection, acute vascular rejection and cellular rejection. The process of transgenesis has helped overcome this obstacle. Transgentic pigs (Sus) have been produced which lower this rejection rate. An hyperacute rejection rate of less than 2% has been seen using trangentic porcine tissue (Harold Y Vanderpool,1999). Using genetic modification the xenogenetic antigens such as the the Galα(1,3)gal antigen is removed by inactivation of the catalyst enzyme GGTA1 from the xenograft cell surface. This increases the acceptability of the graft in humans. In 2015, the first transplantation of an GGTA1 inactivated (GTKO) porcine heart was perfromed. The other xenogenetic antigens, responsible for rejection include a glycan produced by β1,4-N-acetylgalactosaminyltransferase (β4GalNT2) enzyme. Hyper acute rejection is caused by the complement system. Regulating these systems also enhances the acceptability rates of the transplantation.

Human Antibodies

After monoclonal Antibodies were produced in plants, the production of these were also done in mammals, particularly mice (Mus musculus). The concept of unarranged germline-configuration in transgenic monoclonal antibody was suggested by Alt et al in 1985. Reconstruction of the murine heavy chain is performed after the endogenous gene J_H or Cμ have been inactivated. Adittionally, the κ light chain is inavtivated through the Jκ or Cκ genes.These chains are then inserted using pronuclear microinjection.

Monoconal antibodies have a wide application in the field of Medicine and Pharmaceutical industry as the drugs formulated by these are also approved by the US Food and Drug Adminstration (US FDA).The first approved monoclonal Antibody, using transgenic mice was the Panitimumab used for colorectal cancer in the year 2006.

Table 5enlists various monoclonal antibodies produced using transgenic mice currently employed in the treatment of various diseases:

mAb	Target	Indication	US FDA Approval Year
Panitimumab	EGFR	Colorectal Cancer	2006
Ustekinumab	IL-12/23	Psoriasis	2009
Denosumab	RANKL	Bone loss	2010
Ipilimumab	CLTA-4	Metastatic melanoma	2011
Raxibacumab	B.anthracis PA	Anthrax Infection	2012
Evolocumab	PCSK9	High cholestrol	2015
Sarilumab	IL-6R	Rheumatoid Arthritis	2017
Dupilumab	Il-4Rα	Atopic Dermatitis	2017
Cemiplimab	PD-1	Cutaneous squamous	2018

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		cell carcinoma	
Burosumab	FGF23	Hypophosphataemia	2018

TABLE 5:Monoclonal Antibodies produced through genetically modified mice

Spider Silk Production

Spider silk is one of the toughest biopolymer as it has both strength and elasticity. This makes it helpful in a wide array of areas such as the medical field for artificial tendons and ligaments, eye sutures and for jaw repair. Besides this, it can be used for other purposes such as to produce bulletproof vests and better car airbags. Athough it has several applications, the amount of spider silk obtained naturally is minute and hence it is needed to be produced through transgenesis using various animals such as silkworms, mice and goats.

Silkworms (Bombyx mori) is considered the ideal bioreactor for the production of exogenous protein such as spider silk. The silk producing MasP genes in spiders and the FibH in silkworm have similarities to a geat extent which makes it a compatible host for the production of spider silk. The spider silk protein content in silkworms vary between 2-5% at its best and 0.37-0.61% at the least (Jun Xu et al, 2018). This discrepancy is a major hurdle in using this technique for the commercialization of spider silk.

In lactating goats (Capra aegarus) the spider silk genes MaSp1 and MaSp2 genes are expressed in the mammary tissues. This enables us to extract the silk from the milk of the transgenetic organism. Milk protein expression is controlled by the β-casein promoter. The goat can produe upto 2g/L spider silk per day. However, the current issue in using goats for the commercialization of spider silk is the scalability. Hence, lower organisms such as E.coli are used to produce the spider silk which in a 1L fermentor can produce upto 1kg spider silk protirin in a week.

Enhanced Wool Production

Wool is an important fiber in the textile industry used to make various clothing. The primary source for natural wool is sheep (Ovis aries).

Genetic modification of sheeps is conducted inorder to enhance the wool production. This is achieved by introducing ovine β-catenin or ovine insulin like growth factor 1 (IGF1) using pronuclear microinjection along with a skin specific human keratin-14 (k14) promoter. Studies have shown that transgenetic sheeps produced around 6.2% greater clearer fleece (Singhal and Kansara, 2010). This enhanced production of wool has aided the textile industry to meet the growing demand of people.

Nutrition Enriched Cow Milk

Milk is considered to be one of the most nutritious drink which is given to babies right from birth. There are differences in the nutritional content of the human milk and a cow milk. Some component such as the alpha-lactalbumin in the human milk have found to be more nutritonally balanced for babies. Hence, using transgenesis the human alpha-lactalbumin gene was incorporated into cows (Bos taurus). This was first done in 1997, in a cow name Rosie whose milk contained 2.4g of protein per litre of milk. Alpha-lactalbumin has high tryptophan content, which is an essential amino acid, and a high protein content. Tryptophan in normal bovine milk is half that of human milk. The additional benefits of tryptophan include regulation of the sleep-wake cycle along with gutdevelopment and immunity. The tryptophan content in gram/100g of protein of normal cow milk, human milk and milk from transgenetically produced cow is given in figure 8.

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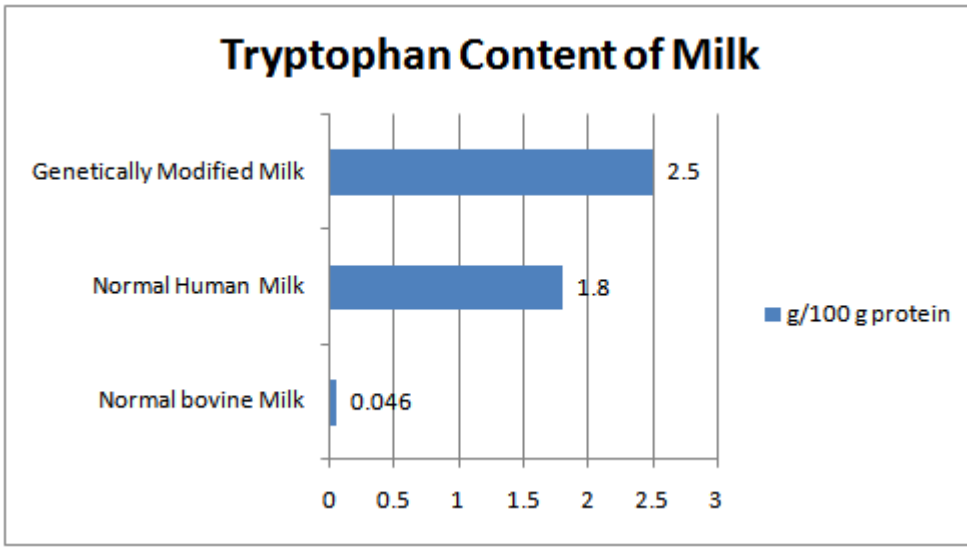

Fig 8: Comparison of the amount of tryptophan in various milk, i.e. Normal Human milk, Normal bovine milk and Genetically modified bovine milk.

In a sudy by Donald K leyman et al in 2018 conducted on infants divided into two categories, it was seen that the infants given alpha-lactalbbumin enriched cow milk in comparison to standard cow milk showed growth patterns similar to the infants who were breastfed.

E) REGULATIONS FOR BIOTECHNOLOGICAL PRODUCTS:

Genetically modified organism have been debated upon since their discovery as they raise questions on the ethical aspects. A group of scientists and common people believe that genetic engineering of organism can be a potential harm to human health and environment. The argument is also extended on the basis of animal cruelty, negative impact on the traditional farming practices, excessive corporate control and the 'unnaturalness' of the technique. Owing to these issues the genetically modified animals are regulated by the Centeral Drug Standard Control Organization (CDSCO) under Ministry of Family Health and Welfare (MoFHW)and the Department of Biotechnology (DBT) under the Ministry of Science and Technology in India. These are regulated under the Drugs and Cosmetic Act, 1945 which are based on the regulations for the production, usage, import, export of micro-organisms genetically engineered organisms 1989 (Rules,1989).These rules are upheld by several guidelines on contained research, biologics, confined field trial, environmental risk asessment etc. The implementation of these rules are overseen by the Ministry of Environment, Forest and Climate Change (MoEFCC), Department of Biotechnology and State Governments though six competent authorities. The six competent authorities and their functions are as given in the table 6.

The sections 8 and 25 of the Environment (Protection) Act,1986 confer to "Regulations of Genome Engineering Technology in India" according to which

i. "Gene Technology" means the application of the gene technique called genetic engineering include self-cloning and deletion as well as cell hybridization.

ii. "Genetic engineering" means the technique by which heritable material, which does not usually occur or will not occur naturally in the organism or cell concerned, generated outside the organism, or the cell is inserted into said cell or organism. It shall also mean the formation of new combinations of genetic material by incorporation of a cell into a host cell, where they occur naturally (self-cloning) as well as modification of an organism or in a cell by deletion and removal of parts of the heritable material.

These rules are applicable to a broad spectrum which include all the Genetic Modified Organisms and their products along with substance, food and products which utilize cells, tissue or organism which have been genetically modified. The

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approvals or prohibitions for GMO's according to the rules of 1989 are as below:

- The import,export,transport,manufacture,process, use or sale of any GMO substance or product without the authorization of GEAC is prohibited.
- Only institutes/labs notified under the EPA,1986 may be allowed to use pathogenic organisms or GMOs or cells for the purpose of gene manipulation
- IBCS must give approval inorder to use GMOs in experiments for educational purposes
- Production in which GMO are used shall be approved by GEAC initially for 4 years, which can then be renewed for every 2 years.
- GEAC may revoke the approvals in case of
  - Harmful effects of the GMO is discovered
  - GMO causes environmental damage which was not apparent at time of approval
  - Any misadherence with the guidelines of GEAC

The guidelines given by CDSCO pertaining to the GMO products in the field of pharmaceutical market include:

- Recombinant DNA safety guideline , 1990
- Guidelines for generating pre-clinical and clinical data for RDNA vaccines, 1999
- Guidelines and Handbook for Institutional Bio-safety committee
- CDSCO guidance for industry, 2008:

a) Capitulation of CTA for Evaluating Safety and Efficacy
b) Requirements for acceptance of New Drugs Approval
c) Post approval changes in biological products: Quality, Safety and Efficacy Documents
d) Establishing the requirements of the Quality Information for Drug Submission for New Drug (Approval: Biotechnological/Biological Products)

- Guidelines and Handbook for Institutional Biosafety Committees (IBSCs), 2011
- Guidelines on Similar Biologics: Regulatory Requirements for Marketing Authorization in India ,2012

CONCLUSION

This report gives a summarized version of the concept of genetically modified organisms, along with thechniques to develop them and their applications in the various fields. Genetic engineering has made huge advancments since the time of its discovery. It has expanded from the field of medicine and agriculture to the industry of textile, industry of chemicals etc. India has also progressed in this field which is evident from the use of crops such as Bt cotton, Bt brinjal in agriculture and the monoclonal antibodies in the field of medicine. Newer technologies for gene editing include Meganucleases, Transcription Activator-Like Effector Nucleases (TALEN). The genome editing technology commonly being used is Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR). The World Health Organisms in 2014 stated "Future genetically modified organisms are likely to include plants with improved resistance against plant disease or drought, crops with increased nutrient levels, fish species with enhanced growth characteristics. For non-food use, they may include plants or animals producing pharmaceutically important proteins such as new vaccines." in respect to the further development of GMO's out of which many we have already seen. The field of Genetic Engineering is yet to see many more advancements and will remain an active field of research for years to come.

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AUTHORITY NAME	ADMINSTRATING AGENCY	FUNCTION
Recombinant DNA Advisory Committee (RDAC)	Department of Biotechnology, Ministry of Science and Technology	Acts as an advisory board which gives recommendations in safety regulations for GMO. They keep updates on biotechnological developments nationally and internationally.
Institutional Biosafety Committee (IBSC)	Set up in registered Institutions, Universities and Private Companies; report to RCGM	This committee is created in each institute carrying genetic manipulation experiments. It comprises of the head of institute, scientists working with DNA, a medical expert and a nominee of the Department of Biotechnology
Review Committee on Genetic Manipulation (RCGM)	Department of Biotechnology, Ministry of Science and Technology	This is a body working under the DBT responsible to ensure the saftey guidelines of ongoing research involving genetic engineering or hazardous microorganism. It includes scientists from Indian Council of Medical Research (ICMR), Indian Council of Agricultural Research (ICAR), and Council of Scientific and Industrial Research (CSIR)
Genetic Engineering Appraisal Committee (GEAC)	Ministry of Environment and Forests and climate change	This is the apex committee of the Ministry of Environent, Forest and Climate Change (MoEFCC). It is responsible for the approval of large scale experiments using genetic engineering.
State Biotechnologiy Coordination Committee (SBCC)	Concerned State Governments	This is a committee constituted in each state involved in experiments related to Genetic engineering.
District Level Committee (DLC)	Concerned State Governments	Similar to SBCC, DLC is constituted in each district where experiments using genetic engineering is performed.

TABLE 6:The six competent authorities and their functions

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