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GENETIC ENGINEERING TECHNIQUES: A REVIEW

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ABSTRACT

Genetic engineering is manipulation of organism genome which does not occur under normal condition and involves insertion of genetic material. The process involves isolation of gene, construction, targeting, transformation, selection, and regeneration which can be done by plasmid and vector method. Genetic engineering technique having widespread application.

INTRODUCTION

Genetic engineering, also called **genetic modification**, is the direct human manipulation of an organism's genetic material in a way that does not occur under natural conditions. It involves the use of recombinant DNA techniques, but does not include traditional animal and plant breeding or mutagenesis. Any organism that is generated using these techniques is considered to be a genetically modified organism. The first organisms genetically engineered were bacteria in 1973 and then mice in 1974. Insulin producing bacteria were commercialized in 1982 and genetically modified food has been sold since 1994[1-3].

The most common form of genetic engineering involves the insertion of new genetic material at an unspecified location in the host genome. This is accomplished by isolating and copying the genetic material of interest, generating a construct containing all the genetic elements for correct expression, and then inserting this construct into the host organism. Other forms of genetic engineering include gene targeting and knocking out specific genes via engineered nuclueases such as zincu finger nucleases or engineered homing endonucleases.

Genetic engineering techniques have been applied in numerous fields including research, biotechnology, and medicine. Medicines such as insulin and human growth hormone are now produced in bacteria, experimental mice such as the oncoumouse and the knockout mouse are being used for research purposes and insect resistant and/or herbicide tolerant crops have been commercialized. Genetically engineered plants and animals capable of producing biotechnology drugs more cheaply than current methods (called pharming) are also being developed and in 2009 the FDA approved the sale of the pharmaceutical protein antithrombin produced in the milk of genetically engineered goats[4].

History

Humans have altered the genomes of species for thousands of years through artificial selection and more recently mutagenesis. Genetic engineering as the direct manipulation of DNA by humans outside breeding and mutations has only existed since the 1970s. The term "genetic engineering" was first coined by Jack Williamson in his science fictionnovel*Dragon's Island*, published in 1951one year before DNA's role in heredity was confirmed by Alfred Hershey and Martha Chase, and two years before James Watson and Francis Crick showed that the DNA molecule has a double-helix structure[9].

In 1972 Paul Berg created the first recombinant DNA molecules by combined DNA from the monkey virus SV40 with that of the lambda virus.In 1973 Herbert Boyer and Stanley Cohen created the first transgenic organism by inserting antibiotic resistance genes into the plasmid

of an *E. coli* bacterium.[5] A year later Rudolf Jaenisch created a transgenic mouse by introducing foreign DNA into its embryo, making it the world's first transgenic animal. In 1976 Genentech, the first genetic engineering company was founded by Herbert Boyer and Robert Swanson and a year later and the company produced a human protein (somatostatin) in *E.coli*. Genentech announced the production of genetically engineered human insulin in1978. In 1980, the U.S. Supreme Court in the Diamond v. Chakrabarty case ruled that genetically altered life could be patented. The insulin produced by bacteria, branded humulin, was approved for release by the Food and Drug Administration in 1982.The first field trials of genetically engineeredplants occurred in France and the USA in 1986, tobacco plants were engineered to be resistant to herbicides. The People's Republic of China was the first country to commercialize transgenic plants, introducing a virus-resistant tobacco in 1992. In 1994 Calguuene attained approval to commercially release the FlavrSavr tomato, a tomato engineered to have a longer shelf life. In 1994, the European Union approved tobacco engineered to be resistant to the herbicide bromoxynil, making it the first genetically engineered crop commercialized in Europe. In 1995, Bt Potato was approved safe by the Environmental Protection Agency, making it the first pesticide producing crop to be approved in the USA. In 2009 11 transgenic crops were grown commercially in 25 countries, the largest of which by area grown were the USA, Brazil, Argentina, India, Canada, China, Paraguay and South Africa.

In 2010, scientists at the J. Craig Venter Institute, announced that they had created the first synthetic bacterial genome, and added it to a cell containing no DNA. The resulting bacterium, named Synthia, was the world's first synthetic life form.

Process[1-3]:

1. Isolating the Gene

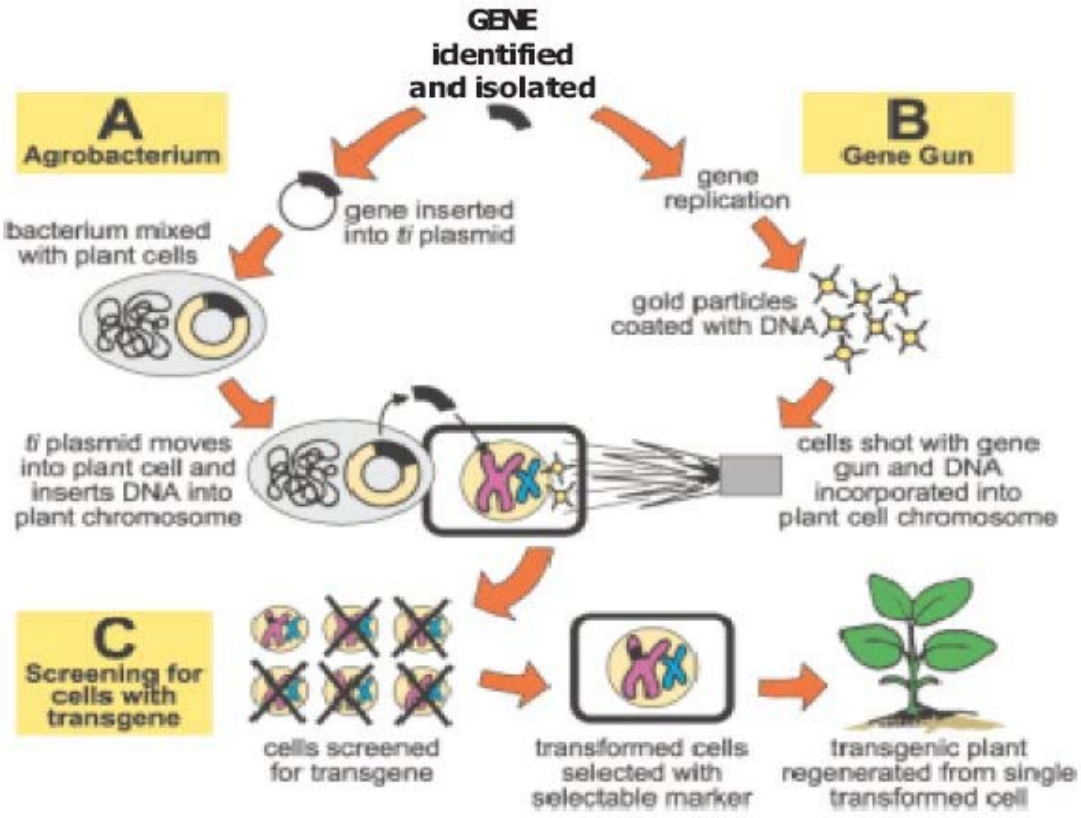

fig .1:Elements of genetic engineering

First, the gene to be inserted into the genetically modified organism must be chosen and isolated. Presently, most genes transferred into plants provide protection against insects or tolerance to herbicides. In animals the majority of genes used are growth hormonegenes. Once chosen the genes must be isolated. This typically involves multiplying the gene using polymerase chain reaction (PCR). If the chosen gene or the donor organism's genome has been well studied it may be present in a genetic library. If the DNA sequence is known, but no copies of the gene are available, it can be artificially synthesized. Once isolated, the gene is inserted into a bacterial plasmid.

2. Construction

The gene to be inserted into the genetically modified organism must be combined with other genetic elements in order for it to work properly. The gene can also be modified at this stage for better expression or effectiveness. As well as the gene to be inserted most constructs contain a promoter and terminator region as well as a selectablemarker gene. The promoter region initiates transcription of the gene and can be used to control the location and level of gene expression, while the terminator region ends transcription. The selectable marker, which

in most cases confers antibiotic resistance to the organism it is expressed in, is needed to determine which cells are transformed with the new gene. The constructs are made using recombinant DNA techniques, such as restriction digests, ligations and molecular cloning.

3. Gene Targeting

The most common form of genetic engineering involves inserting new genetic material randomly within the host genome. Other techniques allow new genetic material to be inserted at a specific location in the host genome or generate mutations at desired genomic loci capable of knocking outendogenous genes. The technique of gene targeting uses homologous recombination to target desired changes to a specific endogenous gene. This tends to occur at a relatively low frequency in plants and animals and generally requires the use of selectable markers. The frequency of gene targeting can be greatly enhanced with the use of engineered nucleases such as zinc finger nucleases, engineered homing endonucleases, or nucleases created from TAL effectors. In addition to enhancing gene targeting, engineered nucleases can also be used to introduce mutations at endogenous genes that generate a gene knockout.

4. Transformation[15]

Fig no.2 Transformation

A. tumefaciens attaching itself to a carrot cell

About 1% of bacteria are naturally able to take up foreign DNA but it can also be induced in other bacteria. Stressing the bacteria for example, with a heat shock or an electric shock, can make the cell membrane permeable to DNA that may then incorporate into their genome or exist as extrachromosomal DNA. DNA is generally inserted into animal cells using microinjection, where it can be injected through the cells nuclear envelope directly into the

nucleus or through the use of viral vectors. In plants the DNA is generally inserted using *Agrobacterium*-mediated recombination or biolistics.

In *Agrobacterium*-mediated recombination the plasmid construct must also contain T-DNA. *Agrobacterium* naturally inserts DNA from a tumor inducing plasmid into any susceptible plant's genome it infects, causing crown gall disease. The T-DNA region of this plasmid is responsible for insertion of the DNA. The genes to be inserted are cloned into a binary vector, which contains T-DNA and can be grown in both *E. Coli* and *Agrobacterium*. Once the binary vector is constructed the plasmid is transformed into *Agrobacterium* containing no plasmids and plant cells are infected. The *Agrobacterium* will then naturally insert the genetic material into the plant cells.

In biolistics particles of gold or tungsten are coated with DNA and then shot into young plant cells or plant embryos. Some genetic material will enter the cells and transform them. This method can be used on plants that are not susceptible to *Agrobacterium* infection and also allows transformation of plant plastids. Another transformation method for plant and animal cells is electroporation. Electroporation involves subjecting the plant or animal cell to an electric shock, which can make the cell membrane permeable to plasmid DNA. In some cases the electroporated cells will incorporate the DNA into their genome. Due to the damage caused to the cells and DNA the transformation efficiency of biolistics and electroporation is lower than agrobacterial mediated transformation and microinjection.

5. Selection

Not all the organism's cells will be transformed with the new genetic material; in most cases a selectable marker is used to differentiate transformed from untransformed cells. If a cell has been successfully transformed with the DNA it will also contain the marker gene. By growing the cells in the presence of an antibiotic or chemical that selects or marks the cells expressing that gene it is possible to separate the transgenic events from the non-transgenic. Another method of screening involves using a DNA probe that will only stick to the inserted gene. A number of strategies have been developed that can remove the selectable marker from the mature transgenic plant.

6. Regeneration

As often only a single cell is transformed with genetic material the organism must be regrown from that single cell. As bacteria consist of a single cell and reproduce clonally regeneration is not necessary. In plants this is accomplished through the use of tissue culture. Each plant species has different requirements for successful regeneration through tissue culture. If

successful an adult plant is produced that contains the transgene in every cell. In animals it is necessary to ensure that the inserted DNA is present in the embryonic stem cells. When the offspring is produced they can be screened for the presence of the gene. All offspring from the first generation will be heterozygous for the inserted gene and must be mated together to produce a homozygous animal.

7. Confirmation

Further tests using PCR, Southern Blots and Bioassays are needed to confirm that the gene is expressed and functions correctly. The organism's offspring are also tested to ensure that the trait can be inherited and that it follows a Mendelian inheritance pattern.

Genetic Engineering Method[1-5]

The methods often used by genetic engineers are many and varied, but generally fall under one of three categories: the **plasmid method**, the **vector method**, and the **biolistic method**.

1. The Plasmid Method

The first technique of genetic engineering, the plasmid method, is the most familiar technique of the three, and is generally used for altering microorganisms such as bacteria. In the plasmid method, a small ring of DNA called a **plasmid** (generally found in bacteria) is placed in a container with special **restriction enzymes** that cut the DNA at a certain recognizable sequence. The same enzyme is then used to treat the DNA sequence to be engineered into the bacteria; this procedure creates "sticky ends" that will fuse together if given the opportunity.

Next, the two separate cut-up DNA sequences are introduced into the same container, where the sticky ends allow them to fuse, thus forming a ring of DNA with additional content. new enzymes are added to help cement the new linkages, and the culture is then separated by molecular weight. Those molecules that weigh the most have successfully incorporated the new DNA, and they are to be preserved[6,7].

The next step involves adding the newly formed plasmids to a culture of live bacteria with known genomes, some of which will take up the free-floating plasmids and begin to express them. In general, the DNA introduced into the plasmid will include not only instructions for making a protein, but also antibiotic-resistance genes. These resistance genes can then be used to separate the bacteria which have taken up the plasmid from those that have not. The scientist simply adds the appropriate antibiotic, and the survivors are virtually guaranteed (barring spontaneous mutations) to possess the new genes.

Next, the scientist allows the successfully altered bacteria to grow and reproduce. They can now be used in experiments or put to work in industry. Furthermore, the bacteria can be

allowed to evolve on their own, with a "selection pressure" provided by the scientist for producing more protein. Because of the power of natural selection, the bacteria produced after many generations will outperform the best of the early generations.

Many people strongly object to the plasmid method of genetic engineering because they fear that the engineered plasmids will be transferred into other bacteria which would cause problems if they expressed the gene. Lateral gene transfer of this type is indeed quite common in bacteria, but in general the bacteria engineered by this method do not come in contact with natural bacteria except in controlled laboratory conditions. Those bacteria that will be used in the wild - for example, those that could clean up oil spills - are generally released for a specific purpose and in a specific area, and they are carefully supervised by scientists.

2. The Vector Method

The second method of genetic engineering is called the vector method. It is similar to the plasmid method, but its products are inserted directly into the genome via a viral vector. The preliminary steps are almost exactly the same: cut the viral DNA and the DNA to be inserted with the same enzyme, combine the two DNA sequences, and separate those that fuse successfully. The only major difference is that portions of the viral DNA, such as those that cause its virulence, must first be removed or the organism to be re-engineered would become ill. This does yield an advantage - removal of large portions of the viral genome allows additional "space" in which to insert new genes.

Once the new viral genomes have been created, they are allowed to synthesize protein coats and then reproduce. Then the viruses are released into the target organism or a specific cellular subset (for example, they may be released into a bacterium via a bacteriophage, or into human lung cells as is hoped can be done for cystic fibrosis patients). The virus infects the target cells, inserting its genome - with the newly engineered portion - into the genome of the target cell, which then begins to express the new sequence.

With vectors as well, marker genes such as genes for antibiotic resistance are often used, giving scientists the ability to test for successful uptake and expression of the new genes. Once again, the engineered organisms can then be used in experiments or in industry. This technique is also being studied as a possible way to cure genetic diseases.

Many people object to this type of genetic engineering as well, citing the unpredictability of the insertion of the new DNA. This could interfere with existing genes' function. In addition, many people are uncomfortable with the idea of deliberately infecting someone with a virus, even a disabled one.

3. The Biolistic Method

The biolistic method, also known as the gene-gun method, is a technique that is most commonly used in engineering plants - for example, when trying to add pesticide resistance to a crop. In this technique, pellets of metal (usually tungsten) coated with the desirable DNA are fired at plant cells. Those cells that take up the DNA (again, this is confirmed with a marker gene) are then allowed to grow into new plants, and may also be cloned to produce more genetically identical crop. Though this technique has less finesse than the others, it has proven quite effective in plant engineering.

Applications of Genetic engineering technique[15-17]:

Genetic engineering has applications in medicine, research, industry and agriculture and can be used on a wide range of plants, animals and micro organism.

1. Medicine

In medicine genetic engineering has been used to mass produce insulin, human growth hormones, follistim (for treating infertility), human albumin, monoclonal antibodies, antihemophilic factors, vaccines and many other drugs. Vaccination generally involves injecting weak live, killed or inactivated forms of viruses or their toxins into the person being immunized. Genetically engineered viruses are being developed that can still confer immunity, but lack the infectioussequences. Mouse hybridomas, cells fused together to create monoclonal antibodies, have been humanised through genetic engineering to create human monoclonal antibodies.

Genetic engineering is used to create animal models of human diseases. Genetically modified mice are the most common genetically engineered animal model. They have been used to study and model cancer (the oncomouse), obesity, heart disease, diabetes, arthritis, substance abuse, anxiety, aging and Parkinson disease. Potential cures can be tested against these mouse models. Also genetically modified pigs have been bred with the aim of increasing the success of pig to human organ transplantation.

The use of rDNA allows scientists to produce many products that were previously available only in limited quantities: for example, insulin, which we referred to earlier. Until the 1980s the only source of insulin for people with diabetes came from animals slaughtered for meat and other purposes[11].

Gene therapy is the genetic engineering of humans by replacing defective human genes with functional copies. This can occur in somatic tissue or germline tissue. If the gene is inserted into the germline tissue it can be passed down to that person's descendants. Gene therapy has

been used to treat patients suffering from immune deficiencies (notably Severe combined immunodeficiency) and trials have been carried out on other genetic disorders. The success of gene therapy so far has been limited and a patient (Jesse Gelsinger) has died during a clinical trial testing a new treatment. There are also ethical concerns should the technology be used not just for treatment, but for enhancement, modification or alteration of a human beings' appearance, adaptability, intelligence, character or behavior. The distinction between cure and enhancement can also be difficult to establish. Transhumanists consider the enhancement of humans desirable.

2. Research

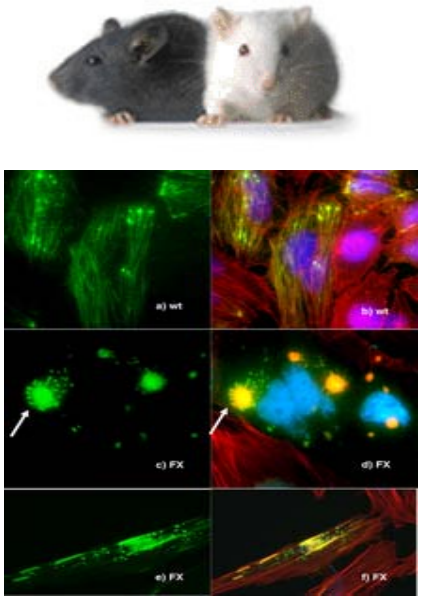

Fig 3: Knockout mice

Human cells in which some proteins are fused with green fluorescent protein to allow them to be visualized. Genetic engineering is an important tool for natural scientists. Genes and other genetic information from a wide range of organisms are transformed into bacteria for storage and modification, creating genetically modified bacteria in the process. Bacteria are cheap, easy to grow, clonal, multiply quickly, relatively easy to transform and can be stored at -80°C almost indefinitely. Once a gene is isolated it can be stored inside the bacteria providing an unlimited supply for research. Organisms are genetically engineered to discover the functions of certain genes. This could be the effect on the phenotype of the organism, where the gene is expressed or what other genes it interacts with. These experiments generally involve loss of function, gain of function, tracking and expression[12].

- **Loss of function experiments**, such as in a gene knockout experiment, in which an organism is engineered to lack the activity of one or more genes. A knockout experiment involves the creation and manipulation of a DNA construct *in vitro*, which, in a simple knockout, consists of a copy of the desired gene, which has been altered such that it is non-functional. Embryonic stem cells incorporate the altered gene, which replaces the already present functional copy. These stem cells are injected into blastocysts, which are implanted into surrogate mothers. This allows the experimenter to analyze the defects caused by this mutation and thereby determine the role of particular genes. It is used especially frequently in developmental biology. Another method, useful in organisms such as Drosophila (fruit fly), is to induce mutations in a large population and then screen the progeny for the desired mutation. A similar process can be used in both plants and prokaryotes[6-8].
- **Gain of function experiments**, the logical counterpart of knockouts. These are sometimes performed in conjunction with knockout experiments to more finely establish the function of the desired gene. The process is much the same as that in knockout engineering, except that the construct is designed to increase the function of the gene, usually by providing extra copies of the gene or inducing synthesis of the protein more frequently.
- **Tracking experiments**, which seek to gain information about the localization and interaction of the desired protein. One way to do this is to replace the wild-type gene with a 'fusion' gene, which is a juxtaposition of the wild-type gene with a reporting element such as green fluorescent protein (GFP) that will allow easy visualization of the products of the genetic modification. While this is a useful technique, the manipulation can destroy the function of the gene, creating secondary effects and possibly calling into question the results of the experiment. More sophisticated techniques are now in development that can track protein products without mitigating their function, such as the addition of small sequences that will serve as binding motifs to monoclonal antibodies.
- **Expression studies** aim to discover where and when specific proteins are produced. In these experiments, the DNA sequence before the DNA that codes for a protein, known as a gene's promoter, is reintroduced into an organism with the protein coding region replaced by a reporter gene such as GFP or an enzyme that catalyzes the production of a dye. Thus the time and place where a particular protein is produced can be observed.

Expression studies can be taken a step further by altering the promoter to find which pieces are crucial for the proper expression of the gene and are actually bound by transcription factor proteins; this process is known as promoter bashing.

3. Industrial Application:

By engineering genes into bacterial plasmids it is possible to create a biological factory that can produce proteins and enzymes. Some genes do not work well in bacteria, so yeast, a eukaryote, can also be used. Bacteria and yeast factories have been used to produce medicines such as insulin, human growth hormone, and vaccines, supplements such as tryptophan, aid in the production of food (chymosin in cheese making) and fuels. Other applications involving genetically engineered bacteria being investigated involve making the bacteria perform tasks outside their natural cycle, such as cleaning up oil spills, carbon and other toxic waste.

4. Agriculture application:

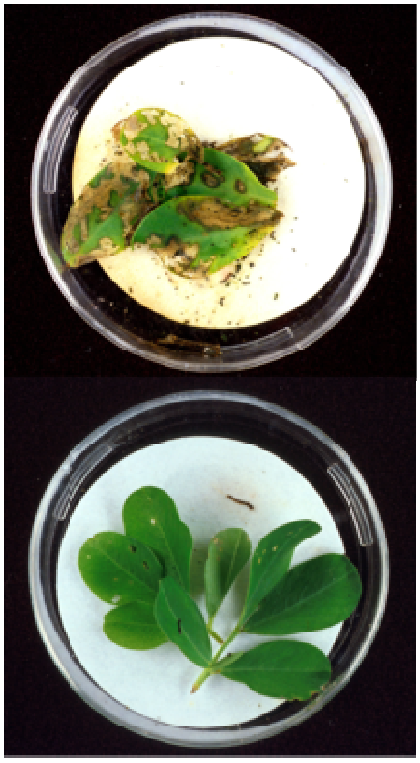

Fig.4: Agriculture: Peanut leaves

Bt-toxins present in peanut leaves (bottom image) protect it from extensive damage caused by European corn borerlarvae (top image). One of the best-known and controversial applications of genetic engineering is the creation of genetically modified food. There are **three** generations of genetically modified crops. The **First** generation crops have been commercialized and most provide protection from insects and/or resistance to herbicides[10].

There are also fungal and virus resistant crops developed or in development. They have been developed to make the insect and weed management of crops easier and can indirectly increase crop yield. The **second** generation of genetically modified crops being developed aim to directly improve yield by improving salt, cold or drought tolerance and to increase the nutritional value of the crops. The **third** generation consists of pharmaceutical crops, crops that contain edible vaccines and other drugs. Some agriculturally important animals have been genetically modified with growth hormones to increase their size while others have been engineered to express drugs and other proteins in their milk. The genetic engineering of agricultural crops can increase the growth rates and resistance to different diseases caused by pathogens and parasites. This is beneficial as it can greatly increase the production of food sources with the usage of fewer resources that would be required to host the world's growing populations. These modified crops would also reduce the usage of chemicals, such as fertilizers and pesticides, and therefore decrease the severity and frequency of the damages produced by these chemical pollution.Ethical and safety concerns have been raised around the use of genetically modified food. A major safety concern relates to the human health implications of eating genetically modified food, in particular whether toxic or allergic reactions could occur. Gene flow into related non-transgenic crops, off target effects on beneficial organisms and the impact on biodiversity are important environmental issues. Ethical concerns involve religious issues, corporate control of the food supply, intellectual property rights and the level of labeling needed on genetically modified products[13,14].

It is now possible to produce plants that will survive freezing temperatures, take longer to ripen, convert atmospheric nitrogen to a form they can use, manufacture their own resistance to pests, and so on. By 1988 scientists had tested more than two dozen kinds of plants engineered to have special properties such as these. Domestic animals have been genetically "engineered" in an inexact way through breeding programs to create more meaty animals, etc., but with genetic engineering, these desirable traits could be guaranteed for each new generation of animal.

Fig.5: Plants are genetically engineered in a laboratory beaker.

5. Applications in Humans

One of the most exciting potential applications of genetic engineering involves the treatment of genetic disorders. Medical scientists now know of about 3,000 disorders that arise because of errors in an individual's DNA. Conditions such as sickle-cell anemia, Tay-Sachs disease, Duchenne muscular dystrophy, Huntington's chorea, cystic fibrosis, and Lesch-Nyhan syndrome are the result of the loss, mistaken insertion, or change of a single nitrogen base in a DNA molecule. Genetic engineering makes it possible for scientists to provide individuals who lack a certain gene with correct copies of that gene. For instance, in 1990 a girl with a disease caused by a defect i n a single gene was treated in the following fashion. Some of her blood was taken, and the missing gene was copied and inserted into her own white blood cells, then the blood was returned to her body. If—and when—that correct gene begins to function, the genetic disorder may be cured. This type of procedure is known as human **gene therapy** (HGT).

CONCLUSION

Gene therapy is used to treat disease by modifying the genetic information in the cells of patient. More than 4000 genetic disease have been characterized and studied. These diseases are caused by lack of production of a single gene or due to production of mutated gene .

Gene therapy include various methods such as **plasmid method,** the **vector method,** and the **biolistic method** and different techniques as recombinant DNA , microinjection, electro chemical poration ,bioballistics etc. Gene therapy techniques are utilized commercially in medicine, research ,industrial, agriculture and pharmaceutical for the production of valuable products. Identification of actual gene responsible for disease, proper gene delivering vectors, complexicity of disease, limited patient Population etc. are major problems for developing of gene therapy. Along with lots of advantages it also has some disadvantages like dramatic fear associated with genetic engineering is the threat that a genetically re-engineered virus could turn out to be extremely virulent, or deadly, and spread.

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