Unit I

SYLLABUS

Recombinant DNA technology, Methods of Gene Transfer in plants, Development of Transgeneics for abiotic and biotic stress tolerance, Tools and techniques used in Agriculture biotechnology.

Invention of Recombinant DNA Technology

Recombinant DNA technology was invented largely through the work of American biochemists Stanley N. Cohen, Herbert W. Boyer, and Paul Berg. In the early 1970s Berg carried out the first successful gene-splicing experiment, in which he combined DNA from two different viruses to form a recombinant DNA molecule. Boyer and Cohen then took the next step of inserting recombinant DNA molecules into bacteria, which replicated, creating many copies of the recombinant molecule. Boyer and Cohen subsequently developed methods for the generation of recombinant plasmids. In 1976, with Robert A. Swanson, Boyer founded the company Genentech, which commercialized Boyer and Cohen's recombinant DNA technology.

In 1968 - prior to the work of Berg, Boyer, and Cohen—Swiss microbiologist Werner Arber discovered restriction enzymes. American microbiologist Hamilton O. Smith subsequently identified type II restriction enzymes. Unlike type I restriction enzymes, which cut DNA at random sites, type II restriction enzymes cleave DNA at specific sites; hence, type II enzymes became important tools in genetic engineering.

I. Plant Breeding

A. Traditional method of improving plants - selective breeding

1. Selection of desired characteristics: yield, palatability, resistance to disease and insects, aesthetic characters

2. Hybridization - bringing together desired genes (by controlled mating) from two or more individuals; results in a combination of desired characters in the offspring - a hybrid

These techniques help farmers to save seed from the best plants to grow next year.

B. Green Revolution - The example of successful crop improvement (dwarf, high yielding wheat varieties)

1. Norman Borlaug, awarded the Nobel Prize in 1970 for his role in the Green Revolution
2. Borlaug is remains concerned about the future noting in particular the international agribusiness control of genetic material

C. Limitations

1. Can only use genes from within one species or several closely related species or wild species, becoming a limitation due to loss of genetic diversity
2. Takes many years to develop an improved variety.

II. Recombinant DNA and the new Genetics:

- A. Introduction

1. In the early 1970's a Moratorium on a certain type of research was called by those doing the work

   - a. legislation was introduced before the US congress which would require congressional approval for these experiments.
     b. involves a newly developed technology of gene manipulation
   - c. experiments were deferred for 18 months so that an assessment could be made regarding the potential danger of the research

2. Technique: ability to construct new combinations of DNA molecules, which do not exist naturally= Recombinant DNA.

III. Combines two different technologies: Restriction Enzymes and Bacterial Plasmids

- A. Restriction Enzymes
  - 1. The real basis for the recombinant DNA techniques: Sequence specific DNases: Recognize short sequences of bases in DNA, and make a double stranded cut in the DNA molecule

    a. function in bacteria- destroy foreign DNA which might enter the cell
    - 1) each bacterium has its own restriction enzymes

    b. each enzyme recognizes only one type of sequence
    - 1) sequence recognized is called a Palindrome

    2) reads the same on the two strands in the opposite direction
    3) example: G A A T T C
    C T T A A G

some enzymes cut straight across, and others make a staggered cut.

G A A T T C
C T T A A G

G AATTC
CTTAA G

Creates fragments of DNA, all with the same overlapping ends.

2. Value of restriction enzymes

a. each enzyme only recognizes the same palindrome regardless of the source of DNA
b. so, fragments, from different sources, produced by the same enzyme, contain the same overlapping ends.
c. treat with ligase = permanently joined together = Recombinant DNA molecule

B. Plasmids

1. Extrachromosomal genetic elements in bacteria

2. Closed circular DNA molecules

3. Replicate independent of the chromosome = many copies/cell

4. Contain genes controlling such things as fertility, and antibiotic resistance

C. Molecular cloning

- 1. The usefulness of the technology combines plasmid biology with restriction enzymes in the following way

a. join a DNA fragment from one source to a bacterial plasmid which has been cut once with the same restriction enzyme

b. plasmid is now called a vector and carries a foreign piece of DNA
c. recombinant plasmid is reintroduced back into a bacterial cell
d. inside the bacterial cell, the plasmid will carry one or more antibiotic resistance factors, so that when plated on a medium containing the antibiotic, only the strain of interest will grow. Plasmid is replicated and the number increased greatly = MOLECULAR CLONING

Unit I
Plant Tissue Culture and its Application

IV. Actual gene transfer to plants:

A. *Agrobacterium tumefaciens* - a bacterium that causes crown gall disease in many plants; unique among pathogens because it causes the disease by transferring bacterial DNA from its plasmid into the plant's chromosome, causes tumors

B. Tissue culture - used to regenerate an entire plant from a single cell; involves growing cells under sterile conditions with different nutrients and hormones; in some plants you can grow entire new plant from one cell:

C. Plant genetic engineering using *Agrobacterium tumefaciens*: method to have one plant species (tobacco - easy to grow, we know a lot about their breeding) express a gene from a different species

- 1. Leaf disk method of plant transformation
  - a. punch out leaf disks

    b. incubate with Agrobacterium culture carrying foreign gene
    c. place disks on culture medium to induce regeneration and to select for **transgenic** plants
    d. root regenerated plants
    e. every cell in the transgenic plant has the foreign gene
    f. these plants pass genes on to their offspring

V. Applications of technology

- A. Getting a gene from one species into another and having it expressed
  - 1. Example - bacteria that makes human insulin, insulin extracted from cows, but gene not human/ now human gene in bacteria to produce insulin

    2. Example - tobacco plant that glows in the dark (luciferin gene from fireflies), transfer of gene not just from one species to another but from one kingdom to another

B. Examples of application of gene technology in agriculture

- 1. Herbicide resistant plants
  - a. in some areas, crops genetically modified for herbicide tolerance could decrease the amount of herbicide used and allow for no-till agriculture, which can minimize erosion
    - 1) Roundup - a herbicide that is effective, non-toxic to animals, short-lived in environment

      2) Research groups developed crop plants resistant to Roundup, notably soybean, corn, tomato, potato, wheat, cotton, alfalfa, etc.

2. Insect resistant plants

- a. Bt toxin gene is cloned from bacteria and expressed in plants to provide resistance from insect without the need for insecticides - data about Bt toxin
- b. currently in crops of corn, corn, cotton
- c. current controversies
  - -effect of pollen from transgenic Bt corn on Monarch butterfly larvae
  - -one form of Bt corn (StarLink) approved for animal feed entered human food products

3. Controlled ripening tomato

a. two varieties of tomato now on the market engineered for delayed ripening

1) one tomato variety has an extra gene - a reverse copy of the gene responsible for an enzyme that breaks down cell walls; as a result, the tomato softens more slowly

2) the other variety has a gene that controls the enzyme necessary for the production of ethylene, one factor that makes a tomato soft

4. Enhanced resistance to viral diseases

- a. Crops bioengineered for pest resistance could increase yield, eliminate the use of several insecticides now derived from fossil fuel, and reduce health risks and groundwater contamination from pesticides
  - 1) used *Agrobacterium* to insert gene from viral protein into plant cells
    2) when regenerated, the plant (tobacco) produced this protein and were more resistant to the virus
    3) currently employed in papaya and squash

5. Production of vitamin A in rice

a. "golden rice" -a transgenic rice in which the genes for the production of vitamin A have been inserted.

VI. Advantages of gene technology

- A. Foreign genes (from outside species) can be introduced
- B. Potentially faster than traditional plant breeding
- C. Specific genes can be transferred; much more control than in traditional plant breeding

VII. Other current and possible benefits of gene technology

- A. Already benefits in human health (human insulin).

B. Genetically engineered fish that grow much faster than wild (growth hormone gene).
C. Recombinant bovine growth hormone already enables cows to use feed more efficiently and produce more milk.
D. Gene Therapy: Use this technology of gene transfer to correct genetic defects; any human diseases known to be due to a single gene defect; use to correct single gene defect and cure disease

- 1. Severe Combined Immune Deficiency. Bubble baby. due to a single gene defect- adenosine deaminase

2. Lesch-Nyhan- severe mental retardation, self destructive behavior. One brain enzyme missing.
3. Cystic Fibrosis- single gene, inhale DNA with normal gene

X. Pros and cons of genetic engineering

- A. Cons- risks and concerns
  - 1. Herbicide-resistance or insect-resistance genes could spread from the engineered crops to wild relatives and create weeds that are especially difficult to control

2. Some scientists fear that the USDA does not require sufficient precautions to prevent the spread of genes from engineered plants to their wild relatives in field trials
3. Some bioengineered products could wipe out the major exports of some developing nations

- a. example: a genetically altered bacterium is under development that produces vanilla flavoring; this could eliminate markets for the vanilla beans, one of Madagascar's major agricultural products

b. bovine growth hormone too expensive for small dairyman, so can't compete with big companies

4. Biological control may solve the problem, cheaper and more effectively, as shown with the work on the cassava mealybug by Dr. Hans Herren, winner of the 1995 World Food Prize

B. Pros

- 1. Almost 100 million people are expected to be added to the world's population each year for the next 30 years
  - a. some believe that without biotechnology, we won't be able to increase the availability of affordable basic food.

b. although biotechnology has potential risks, starvation is worse

Methods of Gene Transfer

Introduction

Genetic transformation is a powerful tool and an important technique for the study of plant functional genomics, i.e., gene discovery, new insights into gene function, and investigation of genetically controlled characteristics. In addition, the function of genes isolated using map-based cloning of mutant alleles has been confirmed by functional complementation using genetic transformation. Furthermore, genetic transformation enables the introduction of foreign genes into crop plants, expeditiously creating new genetically modified organisms. Gene transformation and genetic engineering contribute to an overall increase in crop productivity.

Plant transformation methods

Plant transformation was first described in tobacco in 1984. Since that time, rapid developments in transformation technology have resulted in the genetic modification of many plant species. Methods for introducing diverse genes into plant cells include *Agrobacterium tumefaciens*-mediated transformation, recently reclassified as *Rhizobium radiobacter*, direct gene transfer into protoplasts and particle.

Gene transformation

Several gene transformation techniques utilize DNA uptake into isolated protoplasts mediated by chemical procedures, electroporation, or the use of high-velocity particles (particle bombardment). Direct DNA uptake is useful for both stable transformation and transient gene expression. However, the frequency of stable transformation is low, and it takes a long time to regenerate whole transgenic plants.

Chemical Procedures

Plant protoplasts treated with polyethylene glycol more readily take up DNA from their surrounding medium, and this DNA can be stably integrated into the plant's chromosomal DNA (Mathur & Koncz, 1997). Protoplasts are then cultured under conditions that allowed them to grow cell walls, start dividing to form a callus, develop shoots and roots, and regenerate whole plants.

Electroporation

Plant cell electroporation generally utilizes the protoplast because thick plant cell walls restrict macromolecule movement (Bates, 1999). Electrical pulses are applied to a suspension of protoplasts with DNA placed between electrodes in an electroporation cuvette. Short high-voltage electrical pulses induce the formation of transient micropores in cell membranes allowing DNA to enter the cell and then the nucleus.

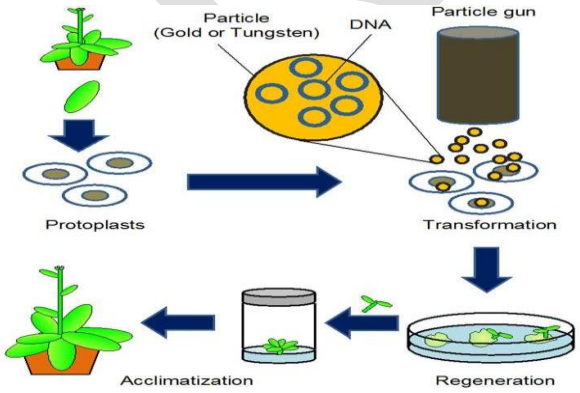


Fig. 1. Plant transformation process using particle bombardment includes the following steps:

(1) Isolate protoplasts from leaf tissues.

(2) Inject DNA-coated particles into the protoplasts using particle gun.

(3) Regenerate into whole plants.

(4) Acclimate the transgenic plants in a greenhouse.

Particle (microprojectile) bombardment

Particle bombardment is a technique used to introduce foreign DNA into plant cells. Gold or tungsten particles (1–2 µm) are coated with the DNA to be used for transformation. The coated particles are loaded into a particle gun and accelerated to high speed either by the electrostatic energy released from a droplet of water exposed to high voltage or using pressurized helium gas; the target could be plant cell suspensions, callus cultures, or tissues. The projectiles penetrate the plant cell walls and membranes. As the microprojectiles enter the cells, transgenes are released from the particle surface for subsequent incorporation into the plant's chromosomal DNA.

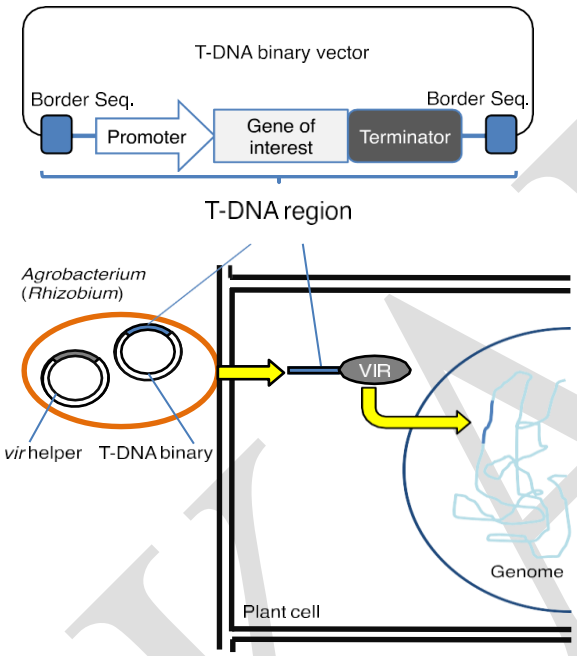


Fig.2. The *Agrobacterium*-mediated transformation process includes the following steps:

(1) Isolate genes of interest from the source organism.
(2) Insert the transgene into the Ti-plasmid.
(3) Introduce the T-DNA containing-plasmid into *Agrobacterium*.
(4) Attach the bacterium to the host cell.
(5) Excise the T-strand from the T-DNA region.
(6) Transfer and integrate T-DNA into the plant genome.

Using *Agrobacterium* for plant transformation

Agrobacterium-mediated transformation is the most commonly used method for plant genetic engineering. The pathogenic soil bacteria *Agrobacterium tumefaciens* that causes crown gall disease has the ability to introduce part of its plasmid DNA (called transfer DNA or T-DNA) into the nuclear genome of infected plant cells (Figure 2).

Transforming *Arabidopsis thaliana*

Arabidopsis thaliana, a small flowering plant, is a model organism widely used in plant molecular biology. The first *in planta* transformation of *Arabidopsis* included the use of tissue culture and plant regeneration. The *Agrobacterium* vacuum and floral dipping are efficient methods to generate transgenic plants. They allow for plant transformation without the need for tissue culture. The floral dipping method markedly advanced the ease of creating *Arabidopsis* transformants, and it is the most widely used transformation method. These methods were later simplified and substantially improved, significantly reduced the required labor, cost, and time, as compared with earlier procedures.

However, these transformation methods have some problems. The floral dipping method involves dipping *Arabidopsis* flower buds into an *Agrobacterium* cell suspension, requiring large volumes of bacterial culture grown in liquid media. The large shakers and centrifuges, necessary to house the media, require sufficient experimental space. These factors limit transformation quantities. Here, we describe an improved method for *Agrobacterium*- mediated transformation that does not require the large volumes of liquid culture necessary for floral dipping.

Improved method for *Agrobacterium*-mediated transformation

A.thaliana can be stably transformed with high efficiency using T-DNA transfer by *Agrobacterium tumefaciens*. *Agrobacterium*-mediated transformation using the floral dipping method is the most widely used method for transforming *Arabidopsis*. We have showed that *A. thaliana* can be transformed by inoculating flower buds with 5 µl of *Agrobacterium* cell suspension, thus avoiding the use of large volumes of *Agrobacterium* culture. Using this floral inoculating method, we obtained 15–50 transgenic plants per three transformed *A. thaliana* plants. The floral inoculating method can be satisfactorily used in subsequent analyses. This simplified method, without floral dipping, offers an equally efficient transformation as previously reported methods. This method reduces overall labor, cost, time, and space. Another important aspect of this modified method is that it allows many independent transformations to be performed at once.

***Agrobacterium* strains**

The *Agrobacterium* strain GV3101 (C58 derivative) is frequently used to transform many binary vectors, e.g., pBI121, pGPTV, pCB301, pCAMBIA, and pGreen, into *Arabidopsis*. It carries rifampicin resistance (10 mg l^{-1}) on the chromosome. On the other hand, LBA4404 is a popular strain for tobacco transformation but is less effective for *Arabidopsis*.

Cell membrane is a sheet like assembly of amphipathic molecules that separate cells from their environment. These physical structures allow only the controlled exchange of materials among the different parts of a cell and with its immediate surroundings. DNA is an anionic polymer, larger molecular weight, hydrophilic and sensitive to nuclease degradation in biological matrices. They cannot easily cross the physical barrier of membrane and enter the cells unless assisted.

Various charged chemical compounds can be used to facilitate DNA transfer directly to the cell. These synthetic compounds are introduced near the vicinity of recipient cells thereby disturbing the cell membranes, widening the pore size and allowing the passage of the DNA into the cell.

An ideal chemical used for DNA transfer should have the ability to-

- Protect DNA against nuclease degradation.
- Transport DNA to the target cells.
- Facilitate transport of DNA across the plasma membrane.
- Promote the import of DNA into the nucleus.

The commonly used methods of chemical transfection use the following,

1. Calcium phosphate
2. DEAE dextran
3. Cationic Lipid
4. Other polymers - poly-L-lysine (PLL), polyphosphoester, chitosan, dendrimers

5-2.1.1. Calcium phosphate mediated DNA transfer

5-2.1.1.1. Historical perspective

The ability of mammalian cells to take up exogenously supplied DNA from their culture medium was first reported by Szybalska and Szybalski (1962).

They used total uncloned genomic DNA to transfect human cells deficient for the enzyme hypoxanthine guanine phosphoribosyl transferase (HPRT). Rare HPRT-positive cells with fragments of DNA containing the functional gene were identified by selection on HAT medium. Till then, the actual mechanism of DNA uptake was not understood. It was later found that successful DNA transfer takes place by the formation of a fine DNA/calcium phosphate co-precipitate, which first settles onto the cells and is then internalized. This technique was first applied by Graham and Van Der Eb in 1973 for the analysis of the infectivity of adenoviral DNA.

5-2.1.1.2. Calcium phosphate transfection

This method is based on the precipitation of plasmid DNA and calcium ions by their interaction.

Inthis method, the precipitates of calcium phosphate and DNA being small and insoluble can be easily adsorbed on the surface of cell. This precipitate is engulfed by cells through endocytosis and the DNA gets integrated into the cell genome resulting in stable or permanent transfection.

Uses

- This method is mainly used in the production of recombinant viral vectors.
- It remains a choice for plasmid DNA transfer in many cell cultures and packaging cell lines. As the precipitate so formed must coat the cells, this method is suitable only for cells growing in monolayer and not for suspension cultures.

Advantages

- Simple and inexpensive
- Applicability to generate stably transfected cell lines
- Highly efficient (cell type dependent) and can be applied to a wide range of cell types.
- Can be used for stable or transient transfection

Disadvantages

- Toxic especially to primary cells
- Slight change in pH, buffer salt concentration and temperature can compromise the efficacy
- Relatively poor transfection efficiency compared to other chemical transfection methods like lipofection.
- Limited by the composition and size of the precipitate.
- Random integration into host cell.

Optimal factors (amount of DNA in the precipitate, the length of time for precipitation reaction and exposure of cells to the precipitate) need to be determined for efficient transfection of the cells.

This technique is simple, expensive and has minimal cytotoxic effect but the low level of transgene expression provoked development of several other methods of transfection.

5-2.1.2. DEAE-Dextran (Diethylaminoethyl Dextran) mediated DNA transfer

- This method was initially reported by Vaheri and Pagano in 1965 for enhancing the viral infectivity of cell but later adapted as a method for plasmid DNA transfer.
- Diethylaminoethyl dextran (DEAE-dextran) is a soluble polycationic carbohydrate that promotes interactions between DNA and endocytotic machinery of the cell.

- In this method, the negatively charged DNA and positively charged DEAE – dextran form aggregates through electrostatic interaction and form apolyplex. A slight excess of DEAE – dextran in mixture results in net positive charge in the DEAE – dextran/ DNA complex formed. These complexes, when added to the cells, bind to the negatively charged plasma membrane and get internalized through endocytosis. Complexed DNA delivery with DEAE-dextran can be improved by osmotic shock using DMSO or glycerol.
- Several parameters such as number of cells, polymer concentration, transfected DNA concentration and duration of transfection should be optimized for a given cell line.

Advantages

- Simple and inexpensive
- More sensitive
- Can be applied to a wide range of cell types
- Can be used for transient transfection.

Disadvantages

- Toxic to cells at high concentrations
- Transfection efficiency varies with cell type
- Can only be used for transient transfection but not for stable transfection
- Typically produces less than 10% delivery in primary cells.

Another polycationic chemical, the detergent Polybrene, has been used for the transfection of Chinese hamster ovary (CHO) cells, which are not amenable to calcium phosphate transfection.

5-2.1.3. Lipofection

- Lipofection is a method of transformation first described in 1965 as a model of cellular membranes using liposomes.
- Liposomes are artificial phospholipid vesicles used for the delivery of a variety of molecules into the cells. They may be multi-lamellar or unilamellar vesicles with a size range of0.1 to 10 micrometer or 20-25 nanometers respectively.
- They can be preloaded with DNA by two common methods- membrane-membrane fusion and endocytosis thus forming DNA- liposome complex. This complex fuses with the protoplasts to release the contents into the cell. Animal cells, plant cells, bacteria, yeast protoplasts are susceptible to lipofection method.

Liposomes can be classified as either cationic liposome or pH-sensitive

5-2.1.3.1. Cationic liposomes

Cationic liposomes are positively charged liposomes which associate with the negatively charged DNA molecules by electrostatic interactions forming a stable complex.

Neutral liposomes are generally used as DNA carriers and helpers of cationic liposomes due to their non-toxic nature and high stability in serum. A positively charged lipid is often mixed with a neutral co-lipid, also called helper lipid to enhance the efficiency of gene transfer by stabilizing the liposome complex (lipoplex). Dioleoylphosphatidyl ethanolamine (DOPE) or dioleoylphosphatidyl choline (DOPC) are some commonly used neutral co-lipids.

- The negatively charged DNA molecule interacts with the positively charged groups of the DOPE or DOPC. DOPE is more efficient and useful than DOPC due to the ability of its inverted hexagonal phase to disrupt the membrane integrity.
- The overall net positive charge allows the close association of the lipoplex with the negatively charged cell membrane followed by uptake into the cell and then into nucleus.
- The lipid: DNA ratio and overall lipid concentration used in the formation of these complexes is particularly required for efficient gene transfer which varies with application.

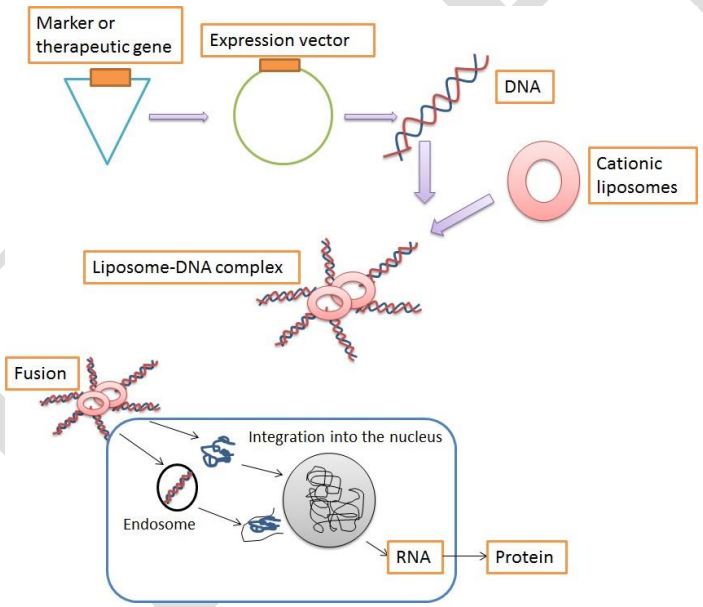
Negatively charged liposomes

Generally pH-sensitive or negatively-charged liposomes are not efficient for gene transfer. They do not form a complex with it due to repulsive electrostatic interactions between the phosphate backbone of DNA and negatively charged groups of the lipids. Some of the DNA molecules get entrapped within the aqueous interior of these liposomes.

However, formation of lipoplex, a complex between DNA and anionic lipidscan occur by using divalent cations (e.g. Ca^{2+}, Mg^{2+}, Mn^{2+}, and Ba^{2+}) which canneutralize the mutual electrostatic repulsion. These anionic lipoplexes comprise anionic lipids, divalent cations, and plasmid DNA which are physiologically safe components.

They are termed as **pH sensitive** due to destabilization at low pH.

The efficiency of both *in vivo* and *in vitro* gene delivery using cationic liposomes is higher thanthat of pH sensitive liposomes. But the cationic liposomes get inactivated and unstable in the presence of serum and exhibit cytotoxicity. Due to reduced toxicity and interference from serum proteins, pH-sensitive liposomes are considered as potential gene delivery vehicles than the cationic liposomes.



Schematic representation of liposome action in gene transfer. ***(Source: Pleyer U, Dannowski H. 2002. Delivery of genes via liposomes to corneal endothelial cells. Drug News Perspect, 15(5): 283)***

In addition, liposomes can be directed to cells using monoclonal antibodies which recognize and bind to the specific surface antigens of cells along with the liposomes. Liposomes can be prevented from destruction by the cell's lysosomes by pre- treating the cells with chemicals such as chloroquine, cytochalasin B, colchicine etc. Liposome mediated transfer into the nucleusis still not completely understood.

Advantages

- Economic
- Efficient delivery of nucleic acids to cells in a culture dish.
- Delivery of the nucleic acids with minimal toxicity.
- Protection of nucleic acids from degradation.
- Measurable changes due to transfected nucleic acids in sequential processes.
- Easy to use, requirement of minimal steps and adaptable to high-throughput systems.

Disadvantages

- It is not applicable to all cell types.
- It fails for the transfection of some cell lines with lipids.

5-2.1.4. Other Methods

Other methods of chemical transfection involve the use of chemicals such as polyethylenimine, chitosan, polyphosphoester, dendrimers.

5-2.1.4.1. Polyethylenimine

- Polyethylenimine (PEI) is a non-degradable, high molecular weight polymer which may accumulate in the body.
- PEI, due to its polycationic nature, condenses with the DNA molecule resulting in the formation of PEI-DNA complex which enters the cell by endocytosis, thus mediating gene transfer.
- PEI exhibit cytotoxicity due to its ability to permeabilize and disrupt cell membranes leading to necrotic cell death.

- The cytotoxicity may be reduced using various methods e.g. PEGylation and conjugation of low molecular weight polyethylenimine with cleavable cross-links such as disulfide bonds in the reducing environment of the cytoplasm.

5-2.1.4.2. Chitosan

- Chitosan, a biodegradable polysaccharide is composed of D-glucosamine repeating units and can be used as a non-viral gene carrier.
- It can efficiently bind and protect DNA from nuclease degradation.
- The biocompatibility and low toxicity profile makes it a safe biomedical material for clinical applications.
- Chitosan DNA nanoparticles can transfect several different cell types with relatively low transfection efficiency.
- Modified chitosans such as trimethylated chitosan and chitosan conjugated with deoxycholic acid have been developed to increase the solubility of chitosan at neutral pH which can efficiently transfect COS-1 cells.
- Chitosans with different molecular weights exhibit different DNA binding affinities. The efficiency of transfection is determined by the particle stability which is one of the rate-limiting steps in the overall transfection process.

5-2.1.4.3. Polyphosphoester

- Polyphosphoesters (PPE) are biocompatible and biodegradable, particularly those having a backbone analogous to nucleic acids and teichoic acids and used in several biomedical applications. They may result in extracellular persistent release of the DNA molecules thus enhancing the expression of transgene in the muscle as compared to naked DNA intake.

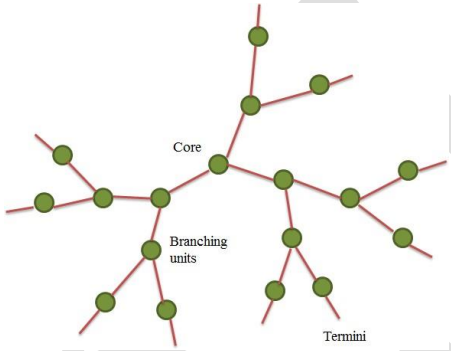
- Several polyphosphoesters with positive charges both in the backbone and in the side chain can be used as non-viral gene carriers.
- They can efficiently bind and protect DNA from nuclease degradation.
- They exhibit a significantly lower cytotoxicity than Poly-L-Lysine or polyethylenimine both *in vitro* and *in vivo.*
- It is a cell type dependent transfection method the efficiency of which can be enhanced using chloroquine.
- The transfection using polyphosphoestersis found to be effective in many cell lines, with some of them comparable to Liposome-mediated transfection.

5-2.1.4.4. Dendrimers

- Dendrimers are a new class of polymeric materials that are highly branched and monodisperse macromolecules. Due to their unique behaviour, they are suitable for a wide range of biomedical applications.
- They have positively charged amino groups (termini) on their surface which interact with the negatively charged phosphate groups of the DNA molecule to form a DNA-dendrimer complex.
- This DNA-dendrimer complex has an overall net positive charge and interacts with negatively charged surface molecules of the cell membrane thus allowing the entry of complex into the cell through non-specific endocytosis.

- Once inside the cell, these complexes are then transported to the endosomes where these are protected from nuclease degradation by being highly condensed within the DNA-dendrimer complex.
- The unprotonated amino groups on the dendrimers at neutral pH can become protonated in the acidic environment of the endosome leading to buffering of the endosome and thus inhibiting pH-dependent endosomal nucleases.

Figure : Structure of a dendrimer.



Development of Transgeneics for abiotic and biotic stress tolerance

The basic concepts of plant stress, acclimation, and adaptation

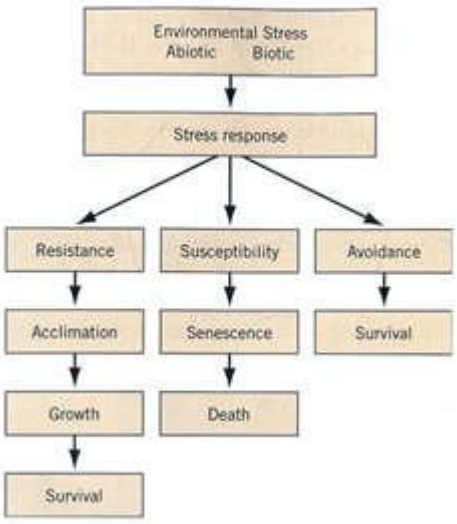
Energy is an absolute requirement for the maintenance of structural organization over the lifetime of the organism. The maintenance of such complex order over time requires a constant through put of energy. The results in a constant flow of energy through all biological organisms, which provides the dynamic driving force for the performance of important maintenance processes such as cellular biosynthesis and transport to maintain its characteristic structure and organization as well as the capacity to replicate and grow. The maintenance of a steady-state results in a meta-stable condition called **homeostasis**.

Environmental modulation of homeostasis defined as biological stress

Any change in the surrounding environment may disrupt homeostasis. Environmental modulation of homeostasis may be defined as **biological stress**. Thus, it follows that **plant stress** implies some adverse effect on the physiology of a plant induced upon a sudden transition from some optimal environmental condition where homeostasis is maintained to some suboptimal condition which disrupts this initial homeostatic state. Thus, plant stress is a relative term since the experimental design to assess the impact of a stress always involves the measurement of a physiological phenomenon in a plant species under a suboptimal, stress condition compared to the measurement of the same physiological phenomenon in the same plant species under optimal conditions.

Plants respond to stress in several different ways

Plant stress can be divided into two primary categories. **Abiotic stress** is a physical (e.g., light, temperature) or chemical insult that the environment may impose on a plant. **Biotic stress** is a biological insult, (e.g., insects, disease) to which a plant may be exposed during its lifetime. Some plants may be injured by a stress, which means that they exhibit one or more metabolic dysfunctions. If the stress is moderate and short term, the injury may be temporary and the plant may recover when the stress is removed. If the stress is severe enough, it may prevent flowering, seed formation, and induce senescence that leads to plant death. Such plants are considered to be **susceptible**. Some plants escape the stress altogether, such as ephemeral, or short-lived, desert plants.



The effect of environmental stress on plant survival

Ephemeral plants germinate, grow, and flower very quickly following seasonal rains. They thus complete their life cycle during a period of adequate moisture and form dormant seeds before the onset of the dry season. In a similar manner, many arctic annuals rapidly complete their life cycle during the short arctic summer and survive over winter in the form of seeds. Because ephemeral plants never really experience the stress of drought or low temperature, these plants survive the environmental stress by **stress avoidance**. Avoidance mechanisms reduce the impact of a stress, even though the stress is present in the environment. Many plants have the capacity to tolerate a particular stress and hence are considered to be **stress resistant**. Stress resistance requires that the organism exhibit the capacity to adjust or to acclimate to the stress. **Stress resistance requires that the organism exhibit the capacity to adjust or to acclimate to the stress.**

A plant stress usually reflects some sudden change in environmental condition. However, in stress-tolerant plant species, exposure to a particular stress leads to **acclimation** to that specific stress in a time-dependent manner. Thus, plant stress and plant acclimation are intimately linked with each other. The stress-induced modulation of homeostasis can be considered as the signal for the plant to initiate processes required for the establishment of a new homeostasis associated with the acclimated state. Plants exhibit stress resistance or stress tolerance because of their genetic capacity to adjust or to acclimate to the stress and establish a new homeostatic state over time. Furthermore, the acclimation process in stress-resistant species is usually reversible upon removal of the external stress.

The establishment of homeostasis associated with the new acclimated state is not the result of a single physiological process but rather the result of many physiological processes that the plant integrates over time, that is, integrates over the acclimation period. Plants usually integrate these physiological processes over a short-term as well as a long-term basis. The *shortterm processes* involved in acclimation can be initiated within seconds or minutes upon exposure to a stress but may be transient in nature. That means that although these processes can be detected very soon after the onset of a stress, their activities also disappear rather rapidly.

As a consequence, the lifetime of these processes is rather short. In contrast, *long-term processes* are less transient and thus usually exhibit a longer lifetime. However, the lifetimes of these processes overlap in time such that the short-term processes usually constitute the initial responses to a stress while the long-term processes are usually detected later in the acclimation process. Such a hierarchy of short- and long-term responses indicates that the attainment of the acclimated state can be considered a complex, time-nested response to a stress. Acclimation usually involves the differential expression of specific sets of genes associated with exposure to a particular stress. The remarkable capacity to *regulate gene expression* in response to environmental change in a time-nested manner is the basis of plant plasticity.

Adaptation and phenotypic plasticity

Plants have various mechanisms that allow them to survive and often prosper in the complex environments in which they live. **Adaptation** to the environment is characterized by genetic changes in the entire population that have been fixed by natural selection over many generations. In contrast, individual plants can also respond to changes in the environment, by directly altering their physiology or morphology to allow them to better survive the new environment. These responses require no new genetic modifications, and if the response of an individual improves with repeated exposure to the new environmental condition then the response is one of acclimation. Such responses are often referred to as **phenotypic plasticity**, and represent nonpermanent changes in the physiology or morphology of the individual that can be reversed if the prevailing environmental conditions change.

Individual plants may also show phenotypic plasticity that allows them to respond to environmental fluctuations

In addition to genetic changes in entire populations, individual plants may also show phenotypic plasticity; they may respond to fluctuations in the environment by directly altering their morphology and physiology. The changes associated with phenotypic plasticity require no new genetic modifications, and many are reversible. Both genetic adaptation and phenotypic plasticity can contribute to the plant's overall tolerance of extremes in their abiotic environment. As a consequence, a plant's physiology and morphology are not static but are very dynamic and responsive to their environment. The ability of biennial plants and winter cultivars of cereal grains to survive over winter is an example of acclimation to low temperature. The process of acclimation to a stress is known as **hardening** and plants that have the capacity to acclimate are commonly referred to as hardy species. In contrast, those plants that exhibit a minimal capacity to acclimate to a specific stress are referred to as no hardy species.

Plants may experience physiological stress when an abiotic factor is deficient or in excess (referred to as an imbalance). The deficiency or excess may be chronic or intermittent. Abiotic conditions to which native plants are adapted may cause physiological stress to non-native plants. Most agricultural crops, for example, are cultivated in regions to which they are not highly adapted. Field crops are estimated to produce only 22% of their genetic potential for yield because of suboptimal climatic and soil conditions.

Imbalances of abiotic factors in the environment cause *primary and secondary effects* in plants.

Primary effects such as reduced water potential and cellular dehydration directly alter the physical and biochemical properties of cells, which then lead to secondary effects. These secondary effects, such as reduced metabolic activity, ion cytotoxicity, and the production of reactive oxygen species, initiate and accelerate the disruption of cellular integrity, and may lead ultimately to cell death. Different abiotic factors may cause similar primary physiological effects because they affect the same cellular processes. This is the case for water deficit, salinity, and freezing, all of which cause reduction in hydrostatic pressure (turgor pressure, Ψp) and cellular dehydration. Secondary physiological effects caused by different abiotic imbalances may overlap substantially. It is evident that imbalances in many abiotic factors reduce cell proliferation, photosynthesis, membrane integrity, and protein stability, and induce production of *reactive oxygen species (ROS)*, oxidative damage, and cell death.

The light-dependent inhibition of photosynthesis

As photoautotrophs, plants are dependent upon – and exquisitely adapted to – visible light for the maintenance of a positive carbon balance through photosynthesis. Higher energy wavelengths of electromagnetic radiation, especially in the ultraviolet range, can inhibit cellular processes by damaging membranes, proteins, and nucleic acids. However, even in the visible range, irradiances far above the light saturation point of photosynthesis cause high light stress, which can disrupt chloroplast structure and reduce photosynthetic rates, a process known as Photoinhibition.

Photoinhibition by high light leads to the production of destructive forms of oxygen

Excess light excitation arriving at the PSII reaction center can lead to its inactivation by the direct damage of the D1 protein. Excess absorption of light energy by photosynthetic pigments also produces excess electrons outpacing the availability of NADP+ to act as an electron sink at PSI. The excess electrons produced by PSI lead to the production of reactive oxygen species (ROS), notably superoxide (O2●-). Superoxide and other ROS are low-molecular-weight molecules that function in signaling and, in excess, cause oxidative damage to proteins, lipids, RNA, and DNA. The oxidative stress generated by excessive ROS destroys cellular and metabolic functions and leads to cell death.

Temperature stress

Mesophytic plants (terrestrial plants adapted to temperate environments that are neither excessively wet nor dry) have a relatively narrow temperature range of about 10°C for optimal growth and development. Outside of this range, varying amounts of damage occur, depending on the magnitude and duration of the temperature fluctuation. In this section we will discuss three types of temperature stress: high temperatures, low temperatures above freezing, and temperatures below freezing. Most actively growing tissues of higher plants are tillable to survive extended exposure to temperatures above 45°C or even short exposure to temperatures of 55°C or above. However, non growing cells or dehydrated tissues (e.g., seeds and pollen) remain viable at much higher temperatures. Pollen grains of some species can survive 70°C and some dry seeds can tolerate temperatures as high as 120°C.

Most plants with access to abundant water are able to maintain leaf temperatures below 45°C by evaporative cooling, even at elevated ambient temperatures. However, high leaf temperatures combined with minimal evaporative cooling causes heat stress. Leaf temperatures can rise to 4 to 5°C above ambient air temperature in bright sunlight near midday, when soil water Deficit causes partial stomatal closure or when high relative humidity reduces the gradient driving evaporative cooling. Increases in leaf temperature during the day can be more pronounced in plants experiencing drought and high irradiance from direct sunlight.

Temperature stress can result in damaged membranes and enzymes

Plant membranes consist of a lipid bilayer interspersed with proteins and sterols, and any abiotic factor that alters membrane properties can disrupt cellular processes. The physical properties of the lipids greatly influence the activities of the integral membrane proteins, including H+-pumping ATPases, carriers, and channel-forming proteins that regulate the transport of ions and other solutes.

High temperatures cause an increase in the fluidity of membrane lipids and a decrease in the strength of hydrogen bonds and electrostatic interactions between polar groups of proteins within the aqueous phase of the membrane. High temperatures thus modify membrane composition and structure, and can cause leakage of ions. High tempeatures can also lead to a loss of the three-dimensional structure required for correct function of enzymes or structural cellular components, thereby leading to loss of proper enzyme structure and activity. Misfolded proteins often aggregate and precipitate, creating serious problems within the cell.

Temperature stress can inhibit photosynthesis

Photosynthesis and respiration are both inhibited by temperature stress. Typically, photosynthetic rates are inhibited by high temperatures to a greater extent than respiratory rates. Although chloroplast enzymes such as rubisco, rubisco activase, NADP-G3P dehydrogenase, and PEP carboxylase become unstable at high temperatures, the temperatures at which these enzymes began to denature and lose activity are distinctly higher than the temperatures at which photosynthetic rates begin to decline. This would indicate that the early stages of heat injury to photosynthesis are more directly related to changes in membrane properties and to uncoupling of the energy transfer mechanisms in chloroplasts.

This imbalance between photosynthesis and respiration is one of the main reasons for the deleterious effects of high temperatures. On an individual plant, leaves growing in the shade have a lower temperature compensation point than leaves that are exposed to the sun (and heat). Reduced photosynthate production may also result from stress-induced stomatal closure, reduction in leaf canopy area, and regulation of assimilate partitioning.

Freezing temperatures cause ice crystal formation and dehydration

Freezing temperatures result in intra- and extracellular ice crystal formation. Intracellular ice formation physically shears membranes and organelles. Extracellular ice crystals, which usually form before the cell contents freeze, may not cause immediate physical damage to cells, but they do cause cellular dehydration. This is because ice formation substantially lowers the water potential (Ψw) in the apoplast, resulting in a gradient from high Ψw in the symplast to low Ψw in the apoplast. Consequently, water moves from the symplast to the apoplast, resulting in cellular dehydration. Cells that are already dehydrated, such as those in seeds and pollen, are relatively less affected by ice crystal formation. Ice usually forms first within the intercellular spaces and in the xylem vessels, along which the ice can quickly propagate. This ice formation is not lethal to hardy plants, and the tissue recovers fully if warmed. However, when plants are exposed to freezing temperatures for an extended period, the growth of extracellular ice crystals leads to physical destruction of membranes and excessive dehydration.

Imbalances in soil minerals

Imbalances in the mineral content of soils can affect plant fitness either indirectly, by affecting plant nutritional status or water uptake, or directly, through toxic effects on plant cells.

Soil mineral content can result in plant stress in various ways

Several anomalies associated with the elemental composition of soils can result in plant stress, including high concentrations of salts (e.g., Na+ and Cl-) and toxic ions (e.g., As and Cd), and low concentrations of essential mineral nutrients, such as Ca2+, Mg2+, N, and P. The term salinity is used to describe excessive accumulation of salt in the soil solution. **Salinity stress** has two components: nonspecific osmotic stress that causes water deficits, and specific ion effects resulting from the accumulation of toxic ions, which disturb nutrient acquisition and result in cytotoxicity. Salt-tolerant plants genetically adapted to salinity are termed *halophytes*, while less salt-tolerant plants that are not adapted to salinity are termed *glycophytes*.

Soil salinity occurs naturally and as the result of improper water management practices

In natural environments, there are many causes of salinity. Terrestrial plants encounter high salinity close to the seashore and in estuaries where seawater and freshwater mix or replace each other with the tides. The movement of seawater upstream into rivers can be substantial, depending on the strength of the tidal surge. Far inland, natural seepage from geologic marine deposits can wash salt into adjoining areas. Evaporation and transpiration remove pure water (as vapor) from the soil, concentrating the salts in the soil solution. Soil salinity is also increased when water droplets from the ocean disperse over land and evaporate.

Human activities also contribute to soil salinization. Improper water management practices associated with intensive agriculture can cause substantial salinization of croplands. In many areas of the world, salinity threatens the production of staple foods. Irrigation water in semiarid and arid regions is often saline. Only halophytes, the most salt-tolerant plants, can tolerate high levels of salts. Glycophytic crops cannot be grown with saline irrigation water. Saline soils are often associated with high concentrations of NaCl, but in some areas Ca2+, Mg2+, and SO4- are also present in high concentrations in saline soils. High Na+ concentrations that occur in sodic soils (soils in which Na+ occupies $\geqslant$10% of the cation exchange capacity) not only injure plants but also degrade the soil structure, decreasing porosity and water permeability. Salt incursion into the soil solution causes water deficits in leaves and inhibits plant growth and metabolism.

High cytosolic Na+ and Cl- denature proteins and destabilize membranes

The most widespread example of a specific ion effect is the cytotoxic accumulation of Na+ and Cl- ions under saline conditions. Under non-saline conditions, the cytosol of higher plant cells contains about 100 mM K+ and less than 10 mM Na+, an ionic environment in which enzymes are optimally functional. In saline environments, cytosolic Na+ and Cl- increase to more than 100 mM, and these ions become cytotoxic. High concentrations of salt cause protein denaturation and membrane destabilization by reducing the hydration of these macromolecules. However, Na+ is a more potent denaturant than K+.

At high concentrations, apoplastic Na+ also competes for sites on transport proteins that are necessary for high-affinity uptake of K+, an essential macronutrient. Further, Na+ displaces Ca2+ from sites on the cell wall, reducing Ca2+ activity in the apoplast and resulting in

greater Na+ influx, presumably through nonselective cation channels. Reduced apoplastic Ca2+ concentrations caused by excess Na+ may also restrict the availability of Ca2+ in the cytosol. Since cytosolic Ca2+ is necessary to activate Na+ detoxification via efflux across the plasma membrane, elevated external Na+ has the ability to block its own detoxification.

Developmental and physiological mechanisms against environmental stress

Plants can modify their life cycles to avoid abiotic stress

One way plants can adapt to extreme environmental conditions is through modification of their life cycles. For example, annual desert plants have short life cycles: they complete them during the periods when water is available, and are dormant (as seeds) during dry periods. Deciduous trees of the temperate zone shed their leaves before the winter so that sensitive leaf tissue is not damaged by cold temperatures. During less predictable stressful events (e.g., a summer of significant but erratic rainfall) the growth habits of some species may confer a degree of tolerance to these conditions. For example, plants that can grow and flower over an extended period (*indeterminate growth*) are often more tolerant to erratic environmental extremes than plants that develop preset numbers of leaves and flower over only very short periods (*determinate growth*).

Phenotypic changes in leaf structure and behavior are important stress responses

Because of their roles in photosynthesis, leaves (or their equivalent) are crucial to the survival of a plant. To function, leaves must be exposed to sunlight and air, but this also makes them particularly vulnerable to environmental extremes. Plants have thus evolved various mechanisms that enable them to avoid or mitigate the effects of abiotic extremes to leaves. Such mechanisms include changes in leaf area, leaf orientation, trichomes, and the cuticle. Turgor reduction is the earliest significant biophysical effect of water deficit. As a result, turgor-dependent processes such as *leaf expansion* and root elongation are the most sensitive to water deficits. When water deficit develops slowly enough to allow changes in developmental processes, it has several effects on growth, one of which is a limitation of leaf expansion. Because leaf expansion depends mostly on cell expansion, the principles that underlie the two processes are similar. Inhibition of cell expansion results in a slowing of leaf expansion early in the development of water deficits.

The resulting smaller leaf area transpires less water, effectively conserving a limited water supply in the soil over a longer period. Altering *leaf shape* is another way that plants can reduce leaf area. Under conditions of water, heat, or salinity extremes, leaves may be narrower or may develop deeper lobes during development. The result is a reduced leaf surface area and therefore, reduced water loss and heat load (defined as amount of heat loss [cooling] required to maintain a leaf temperature close to air temperature). For protection against overheating during water deficit, the leaves of some plants may orient themselves away from the sun. *Leaf orientation* may also change in response to low oxygen availability.

Altered leaf shape can occur in response to environmental changes: leaf from outside (left) and inside (right) of a tree canopy.

Plants can regulate stomatal aperture in response to dehydration stress

The ability to control stomatal aperture allows plants to respond quickly to a changing environment, for example to avoid excessive water loss or limit uptake of liquid or gaseous pollutants through stomata. Stomatal opening and closing is modulated by uptake and loss of water in guard cells, which changes their turgor pressure. Although guard cells can lose turgor as a result of a direct loss of water by evaporation to the atmosphere, stomatal closure in response to dehydration is almost always an active, energy-dependent process rather than a passive one. Abscisic acid (ABA) mediates the solute loss from guard cells that is triggered by a decrease in the water content of the leaf. Plants constantly modulate the concentration and cellular localization of ABA, and this allows them to respond quickly to environmental changes, such as fluctuations in water availability.

Plants adjust osmotically to drying soil by accumulating solutes

Osmotic adjustment is the capacity of plant cells to accumulate solutes and use them to lower Ψw during periods of osmotic stress. The adjustment involves a net increase in solute content per cell that is independent of the volume changes that result from loss of water. The decrease in ΨS (= osmotic potential) is typically limited to about 0.2 to 0.8 MPa, except in plants adapted to extremely dry conditions.

There are two main ways by which **osmotic adjustment** can take place. A plant may *take up ions* from the soil, or *transport ions* from other plant organs to the root, so that the solute concentration of the root cells increases. For example, increased uptake and accumulation of K+ will lead to decreases in ΨS due to the effect of the potassium ions on the osmotic pressure within the cell. This is a common event in saline areas, where ions such as potassium and calcium are readily available to the plant. The accumulation of ions during osmotic adjustment is predominantly restricted to the vacuoles, where the ions are kept out of contact with cytosolic enzymes or organelles.

When ions are compartmentalized in the vacuole, other solutes must accumulate in the cytoplasm to maintain water potential equilibrium within the cell. These solutes are called *compatible solutes* (or *compatible osmolytes*). Compatible solutes are organic compounds that are osmotically active in the cell, but do not destabilize the membrane or interfere with enzyme function, as high concentrations of ions can. Plant cells can hold large concentrations of these compounds without detrimental effects on metabolism. Common compatible solutes include amino acids such as proline, sugar alcohols such as mannitol, and quaternary ammonium compounds such as glycine betaine.

Many plants have the capacity to acclimate to cold temperature

The ability to tolerate freezing temperatures under natural conditions varies greatly among tissues. Seeds and other partially dehydrated tissues, as well as fungal spores, can be kept indefinitely at temperatures near absolute zero (0 K, or -273°C), indicating that these very low temperatures are not intrinsically harmful. Hydrated, vegetative cells can also retain viability at freezing temperatures, provided that ice crystal formation can be restricted to the intercellular spaces and cellular dehydration is not too extreme.

Temperate plants have the capacity for *cold acclimation* – a process whereby exposure to low but nonlethal temperatures (typically above freezing) increases the capacity for low temperature survival. Cold acclimation in nature is induced in the early autumn by exposure to short days and nonfreezing, chilling temperatures, which combine to stop growth. A diffusible factor that promotes acclimation, most likely ABA, moves from leaves via the phloem to overwintering stems. ABA accumulates during cold acclimation and is necessary for this process.

Plants survive freezing temperatures by limiting ice formation

During rapid freezing, the protoplast, including the vacuole, may supercool; that is, the cellular water remains liquid because of its solute content, even at temperatures several degrees below its theoretical freezing point. Supercooling is common to many species of the hardwood forests. Cells can supercool to only about -40°C, the temperature at which ice forms spontaneously. Spontaneous ice formation sets the low-temperature limit at which many alpine and subarctic species that undergo deep supercooling can survive. It may also explain why the altitude of the timberline in mountain ranges is at or near the -40°C minimum isotherm. Several specialized plant proteins, termed **antifreeze proteins**, limit the growth of ice crystals through a mechanism independent of lowering of the freezing point of water. Synthesis of these antifreeze proteins is induced by cold temperatures. The proteins bind to the surfaces of ice crystals to prevent or slow further crystal growth.

Cold-resistant plants tend to have membranes with more unsaturated fatty acids

As temperatures drop, membranes may go through a phase transition from a flexible liquid-crystalline structure to a solid gel structure. The phase transition temperature varies with species (tropical species: 10-12°C; apples: 3-10°C) and the actual lipid composition of the membranes. Chilling-resistant plants tend to have membranes with more unsaturated fatty acids. Chilling-sensitive plants, on the other hand, have a high percentage of saturated fatty acid chains, and membranes with this composition tend to solidify into a semicrystalline state at a temperature well above 0°C. Prolonged exposure to extreme temperatures may result in an altered composition of membrane lipids, a form of acclimation. Certain transmembrane enzymes can alter lipid saturation, by introducing one or more double bonds into fatty acids. This modification lowers the temperature at which the membrane lipids begin a gradual phase change from fluid to semicrystalline form and allows membranes to remain fluid at lower temperatures, thus protecting the plant against damage from chilling.

A large variety of heat shock proteins can be induced by different environmental conditions

Under environmental extremes, protein structure is sensitive to disruption. Plants have several mechanisms to limit or avoid such problems, including osmotic adjustment for maintenance of hydration and chaperone proteins that physically interact with other proteins to facilitate protein folding, reduce misfolding and aggregation, and stabilize protein tertiary structure. In response to sudden 5 to 10°C increases in temperature, plants produce a unique set of chaperone proteins referred to as **heat shock proteins** (HSPs). Cells that have been induced to synthesize HSPs show improved thermal tolerance and can tolerate subsequent exposure to temperatures that otherwise would be lethal. Heat shock proteins are also induced by widely different environmental conditions, including water deficit, ABA treatment, wounding, low temperature, and salinity. Thus, cells that have previously experienced one condition may gain cross-protection against another.

During mild or short-term water shortage, photosynthesis is strongly inhibited, but phloem translocation is unaffected until the shortage becomes severe

Changes in the environment may stimulate shifts in metabolic pathways. When the supply of O_2 is insufficient for aerobic respiration, roots first begin to ferment pyruvate to lactate through the action of lactate dehydrogenase; this recycles NADH to NAD+, allowing the maintenance of ATP production through glycolysis. Production of lactate (lactic acid) lowers the intracellular pH, inhibiting lactate dehydrogenase and activating pyruvate decarboxylase. These changes in enzyme activity quickly lead to a switch from lactate to ethanol production. The net yield of ATP in fermentation is only 2 moles of ATP per mole of hexose sugar catabolized (compared with 36 moles of ATP per mole of hexose respired in aerobic respiration). Thus, injury to root metabolism by O_2 deficiency originates in part from a lack of ATP to drive essential metabolic processes such as root absorption of essential nutrients.

Water shortage decreases both photosynthesis and the consumption of assimilates in the expanding leaves. As a consequence, water shortage indirectly decreases the amount of photosynthate exported from leaves. Because phloem transport depends on pressure gradients, decreased water potential in the phloem during water deficit may inhibit the movement of assimilates. The ability to continue translocating assimilates is a key factor in almost all aspects of plant resistance to drought.

Global warming, and potential climate abnormalities associated with it, crops typically encounter an increased number of abiotic and biotic stress combinations, which severely affect their growth and yield. Concurrent occurrence of abiotic stresses such as drought and heat has been shown to be more destructive to crop production than these stresses occurring separately at different crop growth stages. Abiotic stress conditions such as drought, high and low temperature and salinity are known to influence the occurrence and spread of pathogens, insects, and weeds. They can also result in minor pests to become potential threats in future. These stress conditions also directly affect plant–pest interactions by altering plant physiology and defense responses. Additionally, abiotic stress conditions such as drought enhance competitive interactions of weeds on crops as several weeds exhibit enhanced water use efficiency than crops.

The effect of combined stress factors on crops is not always additive, because the outcome is typically dictated by the nature of interactions between the stress factors. Plants tailor their responses to combined stress factors and exhibit several unique responses, along with other common responses. Therefore, to fully recognize the impact of combined abiotic and biotic stresses on plants, it is important to understand the nature of such interactions. Mittler and colleagues developed a "stress matrix" to compile the interactions among various abiotic and biotic stresses on plant growth and productivity. This matrix illustrates that the stress combinations can have negative as well as positive effects on plants. Therefore, development of plants with enhanced tolerance to combined abiotic and biotic stresses involves identification of physio-morphological traits that are affected by combined stresses.

Based on the currently available studies on the effect of concurrent stresses on plants, this review attempts to improve and amend the current understanding of stress combinations by explaining some fundamental concepts pertaining to them, highlighting their global occurrence and assessing their influence on crop growth.

Examples of Different Stress Combinations Occurring in Nature

Based on the number of interacting factors, stresses can be grouped into three categories: single, multiple individual and combined stresses. A single stress represents only one stress factor affecting plant growth and development, whereas multiple stress represents the impact of two or more stresses occurring at different time periods without any overlap (multiple individual) or occurring concurrently with at least some degree of overlap between them (combined). The co-occurrence of drought and heat stresses during summer is an example of a combined abiotic stress, whereas a bacterial and fungal pathogen attacking a plant at the same time represents a case of combined biotic stress. For example, brown apical necrosis of *JuglansBregia* (walnut) is caused by fungal pathogens *Fusarium*spp. *Alternaria* spp., *Cladosporium* spp., *Colletotrichum* spp., and *Phomopsis* spp., and a bacterium, *Xanthomonasarboricola*.

A first stress factor preceded by another stress factor in sequence may either "endure" (due to priming) or "predispose" the plants to the subsequent stress. For example, drought predisposes *Sorghum bicolor* (sorghum) to *Macrophominaphaseolina*. There are also scenarios where plants are exposed to "repetitive" stresses, where a single or multiple stresses are intervened by short or long recovery periods. For instance, incidences of multiple spells of hot days or multiple occurrences of drought and high temperature at different phenological stages of plants represent repetitive stresses.

Some examples of different stress combinations that are expected to arise due to climate change and their impact on plants. Simultaneously occurring drought and heat stress stands as the most evident stress combination Likewise, plants growing in arid and semi-arid regions often face a combination of salinity and heat stress.

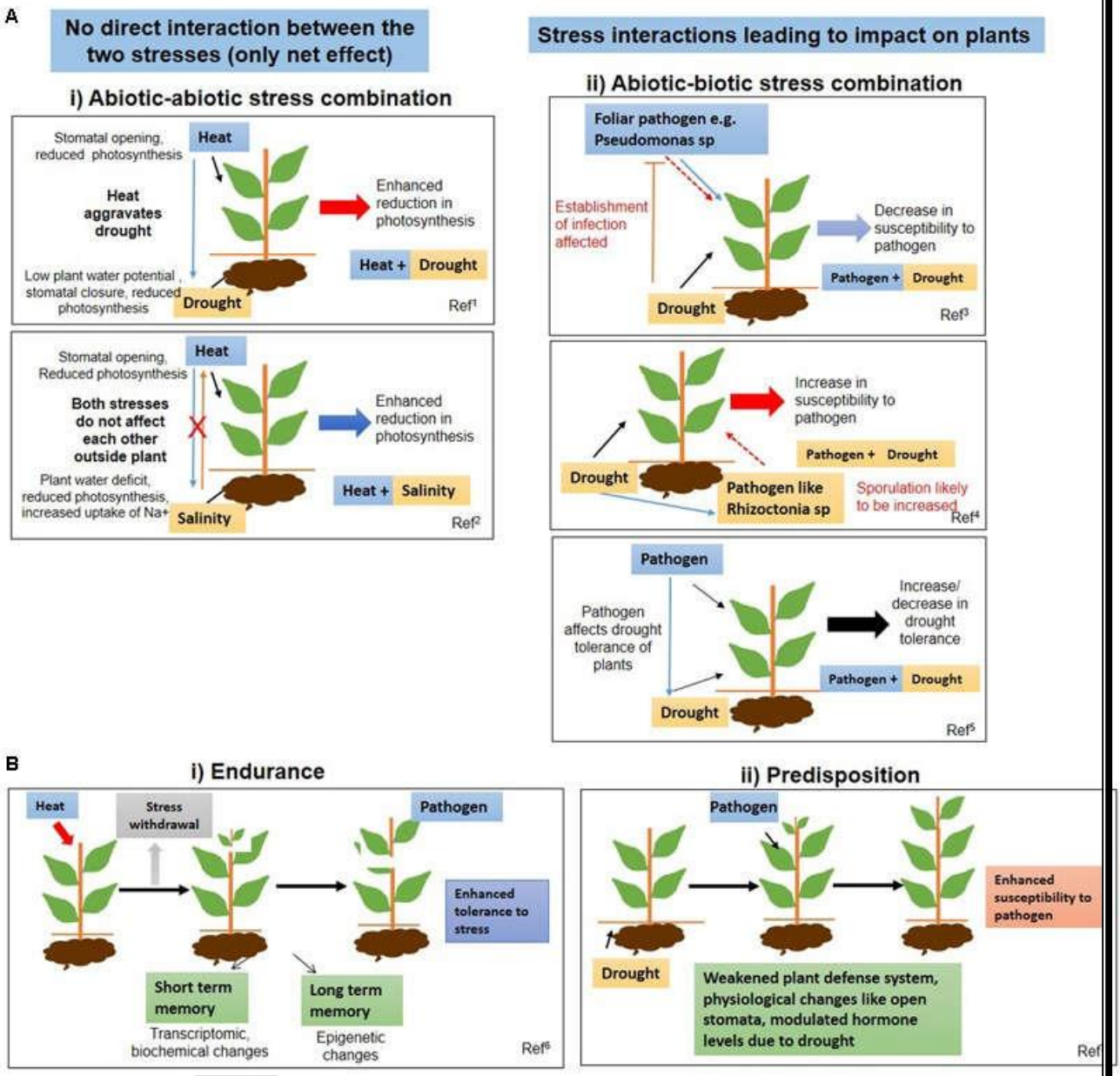
High light stress also often accompanies heat stress. *Vitisvinifera* (grapes) growing in regions characterized by a continental climate, such as North China, face a combination of drought and cold stress which affects their productivity Plants growing in the Mediterranean region encounter combined cold and high light stress. *Triticumaestivum* (winter wheat) is also known to experience a combination of ozone and cold stress which reduces its frost hardiness (Barnes and Davison, 1988). Likewise, salinity combined with ozone stress reduces yields of *Cicer arietinum* (chickpea) and *Oryza sativa* (rice).

Similar to the different abiotic stress combinations, plants also encounter more than one biotic stresses simultaneously or sequentially. Infection by a combination of fungi, bacteria, and viruses are common and are known to cause severe disease symptoms, compared to infections by individual pathogens. Various biotic stress combinations and their impact on plants.
Plants also encounter biotic stressors simultaneously with abiotic stressors. The impact of environmental factors on plant diseases popularly known as the "disease triangle" has always been an important consideration for plant pathologists. Reports have documented the effect of drought or salinity leading to resistance or susceptibility of plants to *Puccinia* spp. (causal agent of rust), *Verticillium*spp. (causal agent of verticillium wilt), *Fusarium* spp. (causal agent of Fusarium wilt), *Pythium* spp. (causal agent of root rot), and *Erysiphe* spp. (causal agent of powdery mildew).The influence of co-occurring, high temperature, or cold stress on increased competitiveness of weeds over crops has also been documented.

Stress interactions as an Important Aspect Governing the Impact of Stress Combinations on Plants crop productivity

Different types of stress interactions can have a range of effects on plants depending on the nature, severity, and duration of the stresses. In case of some abiotic–abiotic and majority of abiotic–biotic stress combinations, interactions not only occur between the plant and the stressors at the plant interface, but also directly between the stressors at or outside the plant interface. In fact, the nature of such interactions between the stressors governs the magnitude of their impact on crop response.

For example, a concurrent heat wave during a drought period may lead to more soil water evaporation resulting in aggravated drought conditions and increased crop yield loss. In addition to this, drought and heat stresses have synergistic effects on plant physiology, resulting in greater negative net impact manifested as drastic yield reduction. Likewise, concurrent drought and weed stress further reduces water availability to crops and subsequently increases the competitiveness of weeds on them.



Schematic representation of effect of stress combination on plants.

(A) Effect of combined stresses on plants is explained by representative examples of heat and drought (abiotic–abiotic stress) and drought and pathogen stress (abiotic–biotic stress) combination.

(i) Depending on the nature of stresses, the two stresses can either not interact physically, but individually affect the plant leading to a net negative impact on plant growth or interact at plant interface and cause a net effect on the plant. Generally, abiotic stress combinations are examples of "only net effects and no stress interactions". For example, simultaneous exposure to heat and salinity leads to enhanced retardation of physiological processes such as photosynthesis.

(ii) Stress interactions are conspicuous in abiotic and biotic stress combinations wherein one stress factor affects the other stress factor *per se*. For example, exposure to combined drought and pathogen stress may result in a complex scenario encompassing an interaction of the two stresses along with the impact of the two stresses on the plant. Depending on the plant pathosystem,
the interaction may lead to enhanced or reduced susceptibility to a particular pathogen. Some pathogens also modulate drought tolerance of the plant.

(B) Effect of multiple individual stresses (sequential stresses) on plants. Sequential stresses may either lead to priming or predisposition of plants to the subsequent stress as explained by examples of heat–pathogen and drought–pathogen stress combinations.

(i) Priming: Exposure of plants to moderate heat stress (indicated by red arrow) may prime the plants to the subsequent pathogen infection. Mild stress can evoke stress memory in the form of epigenetic changes or transcriptomic changes in plants which may last short or longterm, leading to enhanced tolerance of stress to subsequent more severe stresses (same or different stress).

(ii) Predisposition: A pre-occurring drought stress can pre-dispose plants to pathogen infection due to weakened plant defenses or any other metabolic changes occurring due to the drought stress.

In case of stress combinations involving heat and pathogen stress, high temperatures not only affect plants but also pathogens. Temperature is, in fact, one of the most important factors affecting the occurrence of bacterial diseases such as those caused by *Ralstoniasolanacearum* (causal agent of wilt in tomato), *Acidovoraxavenae* (causal agent of seedling blight and bacterial fruit blotch of cucurbits) and *Burkholderiaglumae* (causal agent of bacterial panicle blight in rice) (Kudela, 2009). An increase in temperature modifies the growth rate and reproduction of pathogens (Ladanyi and Horvath, 2010). Temperature also affects the incidence of vector-borne diseases by altering the population development and spread of vectors. Similarly, the effect of salt stress on plant diseases might be the outcome of its modulation on the pathogen virulence, the host physiology and microbial activity in soils. For example, increased incidence of *Fusarium* wilt in *Solanum lycopersicum* (tomato) under salt stress was found to be caused by more sporulation of the fungi under saline conditions.

The combination of two stresses (abiotic–abiotic or abiotic–biotic) does not always lead to negative impact on plants. Some stress combinations negate the effect of each other, leading to a net neutral or positive impact on plants. One stress may also provide endurance to plants against another stress and hence yield is not always negatively impacted. For example, individual drought and ozone stresses are detrimental to the growth of *Medicagotruncatula* (alfalfa), but the combination of drought and ozone results in increased tolerance of plants to the stress combination. High CO_2 has been shown to ameliorate the effect of drought stress in *T. aestivum* and *Poapratensis*(bluegrass). Likewise, an increase in CO_2 level from 350 to 675 ppm favored the competitiveness of the C_3 crop *Glycine max* (soybean) over the C_4 weed *Sorghum halepense*(johnsongrass),*S. lycopersicum* exposed to combined salinity and heat stress performs better than plants subjected to these stresses separately. Ozone treatment also provides enhanced resistance to *Puccinia* spp. in *T. aestivum, Pseudomonas glycinea* (causal agent of bacterial blight) in *G. max* and *Erysiphepolygoni* in *Pisumsativum* (pea).

Some stress combinations exhibit far more complex interactions and their effect on plants are variable. Heat–pathogen and drought–pathogen stress combinations are examples of such complex interactions. For example, with increased temperature, *T. aestivum* and *Avena sativa* (oats) become more susceptible to *Puccinia* spp., but some forage species such as *Cynodondactylon* (Bermuda grass) become more resistant to rust disease. Heat–pathogen and drought–pathogen interactions can be regarded as two agriculturally important stress combinations.

Drought–Pathogen Stress Combination: A Model for Understanding Combined Abiotic–Biotic Stresses

Drought stress interacts with pathogen infection both additively and antagonistically. On the basis of the number of reports of plant diseases being affected by drought stress and the frequency of occurrence of drought stress, this combination can be considered as one of the most important stress combinations affecting crop yields worldwide. Drought stress is reported to enhance the susceptibility of *S. bicolor, T. aestivum, Senecio vulgaris* (groundsel), *Hordeum vulgare* (barley), *Gossypium* spp. (cotton), and *C. arietinum* to *M. phaseolina*, *Puccinia* sp., *Erysiphegraminis* f. sp. *hordei*, *Fusarium oxysporum* f. sp. *vasinfectum*, and *Rhizoctoniabataticola*, respectively. On the other hand, drought stress is reported to provide endurance to tomato, *Medicago sativa* and *Arabidopsis thaliana*against *Botrytis cinerea* (causal agent of gray mold), *Oidiumneolycopersici* (causal agent of powdery mildew), *Verticilliumalboatrum* (causal agent of verticillium wilt), and *Pseudomonas syringae* (causal agent of bacterial speck disease), respectively. In some cases, concurrent pathogen infection helps plants to endure drought stress, resulting in increased yield. For example, infection with *Cucumber mosaic virus* (CMV) led to improved drought tolerance of *Capsicum annum* (pepper), *S. lycopersicum* and *Nicotianatabacum*.

ABIOTIC AND BIOTIC STRESSES

Life took its birth millions of years ago on this planet. Since inception it has undergone tremendous metamorphosis both at structural and functional levels owing to a variety of reasons, be it naturogenic or anthropogenic that ultimately led to the evolutionary process and immense complexity arose in course of the time scale. The changes, which have occurred probably were obviously indispensable, to provide added advantage for survival and perpetuation of the concerned species at a given time. Changes occurred in every aspects of life, which could be conventionally categorized as micro and macro level changes. This is generally designated as evolution, which is continuously taking place. To the contrary of continuous changes, incidence of sudden discrete and discontinuous changes have also accomplished mutation and had been a great source of variation though the quantum of such changes gleaned to be 10^{-7} *in natura*, as evident from fossil data. Such changes are found to be omnipresent and regardless taxa or species, plant or animal, bacteria or virus. The causes and reasons in accomplishing changes in life forms are immense. It was observed at different geological time scale that such changes had different gradients and intensities. In recent times along with naturogenic, a large number of factors of anthropogenic origin became imperative, which plays a key role in driving diverse phenomenon to induce changes in the realms of abiotic and biotic stresses.

ABIOTIC STRESS

In agriculture, among the abiotic stresses, drought, salinity, alkalinity, submergence and mineral toxicity/ deficiencies are serious impediments in lowering both productivity and production. Abiotic stresses like salinity, low temperature and drought in particular- are responsible for most of the reduction that differentiates yield potential from harvestable yield. Salt stress leads to changes in development, growth and productivity and under severe stress survival is threatened. Salinity is a significant complication affecting about a third of the irrigated land and limiting the yield potential of modern cultivars. It has been estimated that salts affect nearly 950 million ha of land globally. Soil salinity constitutes a major abiotic stress in agriculture worldwide and possesses profound detrimental effects on productivity. Conventional selection and breeding techniques have been used to improvise salinity tolerance in crop plants but found to be partially successful in transferring salt tolerance to the target species primarily due to the multigenic origin of the adaptive responses.In India about 8.6 millions of land area is found to be highly prone to salinity. Nearly 20% of the world's cultivated area and nearly 50% of the world's irrigated lands are affected with salinity. About one third of all irrigated land is considered to be affected by salt due to the secondary salinization and it is estimated that about 50% of the arable land is likely to be salinized by 2050. In the coming years, as the quality of irrigation water continues to decline, the problem due to salinity will become ever more acute.

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Over the next three decades, production of food grains in India has to increase at least two million tonnes a year to meet the food demand of the burgeoning population. And this increase has to come from vertical increase in yields from major crops grown since scope of horizontal expansion of cultivable land is virtually nil. One practical means to achieve greater yield is to minimize the abiotic stress associated losses. Attempts to improve yield under stress conditions through conventional plant breeding have been by and large remain unsuccessful, primarily due to the multigenic origin of the adaptive responses. Integration of transgene through genetic engineering / rDNA technology of crop plants appears more feasible. With the advent of genetic transformation techniques based on rDNA technology, it is now possible to dispense alien genes into a recipient plant genome that confers tolerance to abiotic stress including excess salinity/NaCl. Microprojectile bombardment is an important tool in developing transgenic plants especially when the plants are found to be recalcitrant to *Agrobacterium*-mediated transformation. Ability to deliver foreign DNA directly into regenerable cells, tissues, or organs is one of the best methods to develop transgenic lines based on independent transformation events in many agronomic crops through bypassing *Agrobacterium* host specificity and tissue culture-related regeneration difficulties. Absence of biological constraints makes particle bombardment based methodology a versatile and effective transformation method best suited for the genetic manipulation of important crops, not limited by cell type, species or genotype. Keeping this in purview, it is imperative to deploy genetic transformation techniques to meet the future challenges for increased productivity.

BIOTIC STRESS

Among different phyla in the animal kingdom, insects occupy a substantial portion of the biota canvas and displays maximum diversity in comparison to others. Their fossils that are found in the carboniferous period of the geological era that is about 250 million years old indicate their ancient nature. With estimated 5 million species of insects, even to provide a scientific name is a herculean task for the insect taxonomists in contrast to the capability of vertebrate taxonomists, who work with smaller and better-known taxa. Elaborate discussion about insect biodiversity does not come under the purview of this article; however, it is prudent to mention that diversity even in agricultural crop pests is fabulous within the overall ambit of insect biodiversity as evident across different continents over diverse crop plants. Insect pest problems in agriculture are probably as old as agriculture itself.

Following the introduction of high yielding varieties (HYVs) and associated technology, there has been a tremendous increase in the number of insect pests damaging various crops. The extent of damage is influx and a large amount is being spent every year for purchase and application of insecticides in implementing biological control or in adopting modern strategies. The yield is also limited owing to synergistic effect of abiotic along with biotic stresses, wherein understanding the molecular basis of such interactions becomes imperative to unzip the molecular and physiological responses and holds key factor in determining the stress-tolerance strategies which can be adopted. Genetic transformation is one such technique i.e., dove-tailing foreign gene(s) within the genome of plants in productive background efficiently and effectively, which holds immense potential to develop broad-spectrum stress-tolerant plants to avert the impending doom of stress to obtain robust and high-yielding varieties of crop plants.

TRANSGENIC DEVELOPMENT

The importance of genetic transformation in dispensing foreign gene(s) into a desired genetic background in order to modify and agronomically improve upon the crop plants can hardly be over emphasized. It is important because it precisely transfers desired alien gene/s from homo/heterologous sources to a recipient system directly or indirectly through bacterial or viral mediation or through microprojectile based bombardment using DNA coated gold microcarriers acting to move a specific piece of DNA into the protoplasts or intact cells/ tissues. Spectacular advancement has been made in crop genetic modulation through genetic transformation, especially for those characters, which are not found to be amenable through conventional breeding. The transfer of a specific gene or multigenes into a recipient genome has wider application in the genetic manipulation of crop plants. Ideally, a successful plant genetic transformation involves the transfer of foreign gene(s) into a recipient system with its subsequent expression and inheritance of the new DNA in the progenies. Gene transfer methods could be primarily classified as i) vector less system, which notably includes direct transfer of naked DNA through particle bombardment or electroporation of genes through membrane permeabilisation using high molecular weight polyethylene glycol (PEG) and ii) vector based system such as bacteria, viruses, fungi etc. Among various gene trasfer methods, of late, *Agrobacterium*-mediated gene transfer system is gaining preference in rice due to its low cost, convenience and high probability of single copy gene integration with minimum rearrangements. In rice, genotype specificity of the cultivar and strain specificity of *Agrobacterium* seriously hinders the transformation efficiency. Thus, it becomes essential to evolve a race, genotype and strain independent efficient transformation system. Successful developments of transgenics were reported for a wide range of crops like maize, oilseed rape , potato, soyabean, cotton, tomato etc.

Table (1): Some major genes harbouring potential application for abiotic and biotic stress remediation via transgenic research.

AB13	*Arabidopsis thaliana*	Enhanced freezing tolerance	Tamminen*et al.* (2001)
Gs2	Rice	Enhanced salinity resistance and chilling tolerance	Hoshida *et al.* (2000)
atRZ-1a	*Arabidopsis thaliana*	More tolerant to cold	Kim and hunseung (2006)
		Metal toxicity tolerance	
MsFer	*Nicotianatabacum*	Enhanced tolerance of oxidative stress caused by excess Fe	Deak*et al.* (1999)
par β	*Oryzasativa*	Tolerant to higher concentration of Al-toxicity	Conway and Toenniessen (1999)
arsC	*Nicotianatabacum*	Significantly greater Cd tolerance	Dhankher*et al.* (2003)
ALS3	*Arabidopsis thaliana*	Aluminum tolerance	Larsen *et al.* (2005)
ALMT1	*Arabidopsis thaliana*	Aluminum tolerance	Hoekenga*et al.* (2006)
		Biotic	
BOS1	*Arabidopsis thaliana*	Restricts spread of necrotrophic pathogens and provides tolerance to abiotic stresses	Mengiste*et al.* (2003)
MYB4	Rice	Imparts resistance to abiotic (cold, drought, salt, ozone) and biotic stresses (viruses, bacteria, fungi)	Vannini*et al.* (2004, 2006, 2007)
AIM1	Tomato	Integrates responses to biotic and abiotic stresses by modulating ABA signaling and ion fluxes	AbuQamar*et al.* (2009)
PIMP1	Wheat	Modulates response to biotic and abiotic stresses	H.X. Liu *et al.* (2011)
ATAF1	*Arabidopsis thaliana*	Absicisic acid-inducible factor that represses abscisic acid signaling during pathogen infection	Jensen *et al.* (2008)
ATAF2	*Arabidopsis thaliana*	Integrates wounding and pathogen defence responses	Delesser*et al.* (2005)
NTL6	*Arabidopsis thaliana*	A cold-activated inducer of PR gene expression and disease resistance	Seo*et al.* (2010)
NAC6	Rice	An inducer of biotic and abiotic stress responses that has a negative effect on growth	Nakashima *et al.* (2007)
NAC4	Wheat	A transcriptional activator in biotic and abiotic signaling pathways	Xia *et al.* (2010)
DEAR1	*Arabidopsis thaliana*	Negatively regulates components of the salicylic acid-mediated pathogen response	Tsutsui*et al.* (2009)
ERFLP1	Hot pepper	Targets binding domains in genes involved in pathogen defence and salt tolerance	Lee *et al.* (2004)
BIERF1-4	Rice	Positive regulators of disease resistance responses that may also regulate tolerance to abiotic stresses	Cao *et al.* (2006)
ERF3	Soybean	A positive regulator of defence and abiotic stress genes, causing biotic and abiotic stress tolerance when expressed in tobacco	G.Y. Zhang *et al.* (2009)
TS1	Tobacco	Positive regulator of genes in salt tolerance and PR gene-associated pathogen defence pathways	Park *et al.* (2001)

In today's agriculture especially in developed countries transgenic technology holds out as a highly promising area. About 25,000 field trials have been conducted involving 60 species and 10 characters in 45 countries. Now transgenic plants are available, which can withstand biotic stresses like weeds, insects, disease-causing pathogens, viruses, nematodes and abiotic stresses like drought, salinity, alkalinity, oxidative stress, frost and cold, contain improved nutritional qualities like altered fatty acid composition, changed amino acid quantity, delayed fruit ripening, altered flower pigmentation, male sterility, dwarfism, increased photosynthesis efficiency etc [20]. India's joining in growing GM crop for the first time in 2002, the three most populous countries in Asia-China, India and Indonesia accounting for almost 2.5 billion populations are now all set to venture into commercializing GM crops. Also, we cannot increase the area of land under cultivation in order to ensure containing undesirable erosion of natural habitats; the productivity of the current area of cultivated land will have to be enhanced. Transgenic crops are likely to play an increasingly important role in world of agriculture in the coming decades. To feed the ever-increasing population more and more food needs to be produced from dwindling resources like less land, water and energy and declining bio resources by increasing the productivity of agricultural crops to considerable extent.

An eco-friendly approach in management of insect pest is the use of soil bacterium *Bacillus thuringiensis,*which has attracted attention of both microbiologists and entomologists since long back. Use of spores from naturally occurring *B. thuringiensis* in the fermented forms was found to be promising in controlling insects. The first *B.thuringiensis*strain was commercialized and marketed as 'thuricide', which were used as spray and in the form of aerosols. This free-living bacterium is abundant in the soil environment and different strains are available worldwide. Bt proteins possess specific insecticidal action towards certain insect orders and are reported to be safe to non-target insects, birds and mammals. Delta (δ) -endotoxins possesses two properties that are essential for toxins used in transgenic crops. These are highly toxic to certain insect pests, and reported to be safe for human consumption [21]. Upon ingestion by susceptible host, endospore sporulates and produces an array of toxins, including crystalline delta (δ) toxin.

In presence of proteases and at high pH of 7.5-8.0 in insect gut environment, the protoxins (65-70 kDa) cleave to yield an active toxin, which percolates through the peritropic membrane and bind to specific receptors on the apical microvillar brush border membrane. In highly sensitive insect species, the microvilli lose their characteristic structure within minutes of insertion and the cells become vacuolated and ultimately burst. The alkaline gut juice begins to leak out into the haemocoel. Consequently, the haemocoel pH rises causing paralysis, which leads to inhibition in feeding and eventual death of the insect [22]. Based on structure, antigenic properties and activity spectrum, crystal proteins and their genes have been classified into four major groups: cry I (Lepidoptera specific), cry II (Lepidoptera and Diptera specific), cry III (Coleoptera specific) and cry IV (Diptera specific). The Lepidopteran-active cryI genes are the most thoroughly studied group among all δ-endotoxin genes. The cry I genes encode proteinaceousprotoxins of 130-140 kDa, which were found to be localized within bacterial spores as bipyramidal crystal inclusions. The δ-endotoxins and associated peptides remain present in less quantities, however, may super code amounts greater than 20% of the weight of the spore [23]. Despite the unprecedented success Bt products accounts only about 1 % of the total 'agrochemical market'.

Unit II
SYLLABUS

Heterosis and Apomixis and their significance, Mutation and Polyploidy in crop improvement, Molecular markers, Marker assisted breeding, QTL mapping, Origin, evolution and cultivation practices of the major crop plants.

Heterosis, **hybrid vigor**, or **outbreeding enhancement** is the improved or increased function of any biological quality in a hybrid offspring. An offspring is heterotic if its traits are enhanced as a result of mixing the genetic contributions of its parents. These effects can be due to Mendelian or non-Mendelian inheritance.
In proposing the term *heterosis* to replace the older term **heterozygosis**, G.H. Shull aimed to avoid limiting the term to the effects that can be explained by heterozygosity in Mendelian inheritance.

Heterosis is often discussed as the opposite of inbreeding depression although differences in these two concepts can be seen in evolutionary considerations such as the role of genetic variation or the effects of genetic drift in small populations on these concepts. Inbreeding depression occurs when related parents have children with traits that negatively influence their fitness largely due to homozygosity. In such instances, outcrossing should result in heterosis.

Not all outcrosses result in heterosis. For example, when a hybrid inherits traits from its parents that are not fully compatible, fitness can be reduced. This is a form of outbreeding depression.

Dominance versus overdominance is a scientific controversy in the field of genetics that has persisted for more than a century.[2] These two alternative hypotheses were first stated in 1908.

When a population is small or inbred, it tends to lose genetic diversity. Inbreeding depression is the loss of fitness due to loss of genetic diversity. Inbred strains tend to be homozygous for recessive alleles that are mildly harmful (or produce a trait that is undesirable from the standpoint of the breeder). Heterosis or hybrid vigor, on the other hand, is the tendency of outbred strains to exceed both inbred parents in fitness.

Selective breeding of plants and animals, including hybridization, began long before there was an understanding of underlying scientific principles. In the early 20th century, after Mendel's laws came to be understood and accepted, geneticists undertook to explain the superior vigor of many plant hybrids. Two competing hypotheses, which are not mutually exclusive, were developed.

- **Dominance hypothesis**. The dominance hypothesis attributes the superiority of hybrids to the suppression of undesirable recessive alleles from one parent by dominant alleles from the other. It attributes the poor performance of inbred strains to loss of genetic diversity, with the strains becoming purely homozygous at many loci. The dominance hypothesis was first expressed in 1908 by the geneticist Charles Davenport.[4] Under the dominance hypothesis, deleterious alleles are expected to be maintained in a random-mating population at a selection–mutation balance that would

depend on the rate of mutation, the effect of the alleles and the degree to which alleles are expressed in heterozygotes.

- **Overdominance hypothesis**. Certain combinations of alleles that can be obtained by crossing two inbred strains are advantageous in the heterozygote. The overdominance hypothesis attributes the heterozygote advantage to the survival of many alleles that are recessive and harmful in homozygotes. It attributes the poor performance of inbred strains to a high percentage of these harmful recessives. The overdominance hypothesis was developed independently by Edward M. East (1908) and George Shull (1908). Genetic variation at an overdominant locus is expected to be maintained by balancing selection. The high fitness of heterozygous genotypes favours the persistence of an allelic polymorphism in the population.

Dominance and overdominance have different consequences for the gene expression profile of the individuals. If overdominance is the main cause for the fitness advantages of heterosis, then there should be an over-expression of certain genes in the heterozygous offspring compared to the homozygous parents. On the other hand, if dominance is the cause, fewer genes should be under-expressed in the heterozygous offspring compared to the parents. Furthermore, for any given gene, the expression should be comparable to the one observed in the fitter of the two parents.

Apomixis – classification and significance in plant breeding

Apomixis, derived from two Greek word "APO" (away from) and "mixis" (act of mixing or mingling). It refers to the occurrence of an sexual reproductive process in the place of normal sexual processes involving reduction division and fertilization. In other words apomixis is a type of reproduction in which sexual organs of related structures take part but seeds are formed without union of gametes. Seeds formed in this way are vegetative in origin. When apomixis is the only method of reproduction in a plant species, it is known as obligate apomixis. On the other hand, if gametic and apomictic reproduction occurs in the same plant, it is known as facultative apomixis. The first discovery of this phenomenon is credited to Leuwenhock as early as 1719 in Citrus seeds. Apomixis is widely distributed among higher plants. More than 300 species belonging to 35 families are apomictic. It is most common in Gramineae, Compositae, Rosaceae and Rutaceae. Among the major cereals maize, wheat and pearl millet have apomictic relatives. Apomixis Long back, Winkler (1908) defined apomixis as "the substitution for sexual reproduction or another asexual reproductive process that does not involve nuclear or cellular fusion (i.e. fertilization)". Stebbins (1914) and later Nygren (1954) presented an excellent review on apomixis in angiosperms, which can be referred to for greater details. Here, a brief account of apomixis, is furnished only from the point of view of breeding. Types of apomixis Mainly four types of apomixis phenomenon are suggested by Maheshwari (1954) 1. Recurrent Apomixis An embryo sac develops from the MMC or megaspore mother cell (archesporial cell) where meiosis is disturbed (sporogenesis failed) or from some adjoining cell (in that case MMC disintegrates). Consequently, the egg-cell is diploid. The embryo subsequently develops directly from the diploid egg-cell without fertilization. Somatic apospory, diploid parthenogenesis and diploid apogamy are recurrent apomixis. However, diploid parthenogenesis / apogamy occur only in aposporic (somatic) embryo-sacs. Therefore, it is the somatic or diploid aposory that constitutes the recurrent apomixis. Such apomixis occurs in some species of Crepis, Taraxacum, Paa (blue grass), and

Allium (onion) without the stimulus of pollination. Malus (apple), and Rudbeckia where pollination appears to be necessary, either to stimulate embryo development or to produce a viable endosperm.

2. Non -recurrent Apomixis An embryo arises directly from normal egg-cell (n) without fertilization. Since an eggcell is haploid, the resulting embryo will also be haploid. Haploid parthenogenesis and haploid apogamy, and androgamy fall in this category. Such types of apomixis are of rare occurrence. They do not perpetuate and are primarily of genetic interest as in com.

3. Adventive Embryony Embryos arise from a cell or a group of cells either in the nucellus or in the integuments, e.g. in oranges and roses. Since it takes place outside the embryo sac, it is not grouped with recurrent apomixis, though this is regenerated with the accuracy. In addition to such embryos, regular embryo within the embryo sac may also develop simultaneously, thus giving rise to poly-embryony condition, as in Citrus, Opuntia.

4. Vegetative apomixis In some cases like Poa bulbosa and some Allium, Agave and grass species, vegetative buds or bulbils, instead of flowers are produced in the inflorescence. They can also be reproduced without difficulty. However, Russian workers do not group this type of vegetative reproduction with apomixis. Now, different apomictic phenomena in each of the recurrent and non-recurrent apomicts are considered in relation to the development of the embryo sac or embryo.

Advantages of apomixis in plant breeding The two sexual processes, self-and crossfertilization, followed by segregation, tend to alter the genetic composition of plants reproduced through amphimixis. Inbreeding and uncontrolled out breeding also tend to break heterozygote superiority in such plants. On the contrary, apmicts tend to conserve the genetic structure of their carriers. They are also capable of maintaining heterozygote advantages generation after generation. Therefore, such a mechanism might offer a great advantage in plant breeding where genetic uniformity maintained over generation for both homozygosity (in varieties of seIfers), and heterozygosity (in hybrids of both seIfers and outbreeders) is the choicest goal. Additionally, apomixis may also affect an efficient exploitation of maternal influence, if any, reflecting in the resultant progenies, early or delayed because it causes the perpetuation of only maternal individuals and maternal properties due to prohibition of fertilization. Maternal effects are most common in horticultural crops, particularly fruit trees and ornamental plants.

Thus, in short the benefits of apomixis, insofar as their utility in plant breeding is concerned, are:

1. Rapid multiplication of genetically uniform individuals can be achieved without risk of segregation.

2. Heterosis or hybrid vigour can permanently be fixed in crop plants, thus no problem for recurring seed production of F 1 hybrids.

3. Efficient exploitation of maternal effect, if present, is possible from generation to generation.

4. Homozygous inbred lines, as in corn, can be rapidly developed as they produce sectors of diploid tissues and occasional fertile gametes and seeds.

Exploitation of apomixis in crop improvement:

Class: I M.Sc. BT Course Name: Agricultural Biotechnology
Course Code: 19BTP205B Batch: 2019

Unit II

Genetic and Molecular basis

The use of apomixis in crops in a follow-up process, after a variety or hybrid is evolved, as reflected by the benefits it renders. Therefore, our aim in this section is to deal with only apomixis as a tool to plant breeding. With a view to exploit apomixis in sexual crops, it needs to detect and identify an apomictic phenomenon, occurring spontaneously in any plant, or, to incorporate it artificially, perhaps through hybridization between apomicts and amphimicts.

Detection of apomixis:

Positive evidence for the presence or absence of apomixis can be obtained only from an intensive screening of a large number of plants in a variety/hybrid. The screening involves a careful and systematic tracing of steps for the development of embryo-sac and embryo, through microtomy of ovule, right from megaspores to embryonic development. as such, therefore, it is a most tedious job requiring a lot of patience and persistence indeed.

It should however be noted that it is only recurrent apomixis, namely diploid forms of apospory / parthenogenesis / apogamy / adventive embryony and vegetative propagation which are beneficial for plant breeding purposes. The simple reason being that it is these which produce viable diploid embryos without fertilization and thus can continue to perpetuate over generations. Nonrecurrent apomixis are of academic use.

Maintenance of apomixis:

Once an apomict plant is detected its inheritance should promptly be studied by crossing a half or few flowers with the pollen obtained from normal plants and going through the segregation pattern in F2 and onward generations. The remaining flowers may thoroughly be checked and seeds collected on maturity. The true nature of such plants would become distinct only after progeny tests. A true apomictic plant will automatically produce mother apomictic progenies which can be maintained without difficulty.

Transfer of apomixes:

With regard to transfer of apomixis, substantial evidence is available for the hybrid origin of many of the apomicts. Nevertheless, there is no evidence at all the hybridization by itself can induce apomixis (Stebbins, 1950). Situation is further aggravated by the unstable nature of apomicts since there is every likelihood of the breaking down of interacting gene complexes conditioning apomixis, as stated earlier. Therefore, possibilities of introducing apomixis in non-apomicts are the least but not totally absent.

MUTATION

Mutation, i.e. the heritable change to an individual's genetic makeup, results in new traits which are passed on from parent to offspring and thereby, drives evolution. In nature, mutations are caused by errors in the replication of deoxyribonucleic acid (DNA). This hereditary material could also be changed due to exposure to surroundings' natural radiations. A resulting modified individual is then known as a spontaneous mutant. Mutation is the underlying cause of evolution as an individual with a novel trait may be preferentially selected for in nature – because its superior fitness arising from novel (mutant) adaptive features – or artificially by man – because of the desirability of the novelty. Following the discoveries of X-rays by Roentgen in 1895; radioactivity by Becquerel in 1896; and radioactive elements by Marie and Pierre Curie in 1898, it was shortly afterwards demonstrated that radiation caused mutations in fruit flies (Muller, 1927) and in the crop

plants – maize and barley (Staedler, 1928). The subsequent rapid and widespread adoption of induced mutations as a crop improvement tool derives directly from these pioneering discoveries. It became evident that man did not have to wait for chance discoveries of desirable off-type plants as was the case for our forebear huntergatherers. Man could, in fact, induce mutations at will

Mutation breeding has witnessed spectacular successes since the release of the first induced mutant variety – a light green mutant of tobacco released in Indonesia in the mid-1930s. Easy targets for plant mutation breeding are annual, inbred, seed propagated crops: seeds are ideal for mutation induction and short life-cycles mean mutant generations can be produced quickly and desirable mutant lines can be developed into varieties rapidly. Thus, early successes were made in crops such as rice, barley and tobacco and these have been sustained ever since.

Next up are the annual, outbred seed crops, these have a slightly more complex breeding system, but nevertheless early successes in developing mutant varieties were reported and there has been a continual production of new mutant varieties in crops such as maize by the late 60s in various countries. More problematic are the vegetatively propagated crops which have lagged, behind seed propagated crops in mutation breeding. This group was targeted by Frantisek Novak circa 1980s.

Working at the FAO/IAEA's Plant Breeding and Genetics Laboratory, Novak and his team pioneered tissue culture methods needed for banana micro-propagation. Micro-propagation is essential as it allows large numbers of cuttings to be produced for both mutation induction and subsequent mutant lines development. The vegetatively propagated crops are 2 currently undergoing a renaissance with respect to plant mutation breeding as numerous biotechnologies can be applied for efficient mutagenesis and mutant screening, particularly tissue culture techniques. And last, but not least, are the perennial crops, these naturally have long juvenile stages and have been neglected as it takes many years for them to bear fruit.

A further hindrance in plant mutation breeding has been the reliance on phenotypic screening which is normally applied in the second generation after mutation induction at the earliest. This modus operandi is changing with the emergence of DNA analytical tools. Genotypic screening has the potential to accelerate both mutation detection and mutant line development. This can be applied to all crops, but has special relevance to (orphaned) perennial and plantation crops such as oil palm, cocoa, rubber, tea and coffee.

1.1.TRADIATION TYPES AND SERVICES

This chapter is an update, largely based on the chapter on Mutagenic Radiation presented in the second edition of the Manual on Mutation Breeding published in 1977. Physical mutagens comprise all nuclear radiations and sources of radio-activity including ultraviolet light (a non-ionising radiation), several types of ionizing radiations, namely X- and gamma-rays, alpha and beta particles, protons and neutrons. An overview of the main physical mutagens used in plant mutation breeding is presented here, covering their physical characteristics, their mode of action and all general principles and consideration on how they may be applied for mutation induction in plants. Several types of ionizing radiation are available for plant mutation induction. Each of these has the common feature of releasing ionizing energy. However, there are several differences among ionizing radiations regarding the energy

deployed the penetrating capability and the level of hazard involved for operators. X-rays are known to originate from electrons and not from nuclear energy. Like gamma-rays and ultraviolet (UV) light, X-rays are electro-magnetic radiations emitted as quanta, their difference is based on the wavelengths; 0.001 – 10 nm for gamma and X-rays compared to 2000 – 3000 nm for UV light.

In an X-ray machine, electrons are electrically accelerated in a high vacuum and then stopped abruptly by striking a target, e.g. a tungsten, gold or molybdenum barrier resulting in the emission of radiation. For mutation induction hard X-rays (short wavelength) are usually preferred since their penetration is greater than soft X-rays (which have a longer wavelength). The shortest wavelength emitted (except for constant potential machines) is related to the peak operating voltage (kVp) of the X-ray tube, the higher the kVp, the shorter the wavelength.

Specific filters, e.g. aluminium filter: 0.5nm, are often used in hard X-ray production to absorb unwanted soft radiation. The kVp, milliamperes (mA), thickness and type of filter, distance of tube to target, dose and dose rate affect the results and should always be recorded (Mehta and Parker, 2011).

Gamma-rays:

In general, gamma-rays emitted by decay of an unstable nucleus of an atom, have a shorter wavelength and therefore possess more energy per photon than X-rays. Mono energetic gamma radiation is usually obtained from radio-isotopes, in contrast to X- 6 rays. A gamma irradiation facility can be used in a similar manner as an X-ray machine for acute or semi–acute exposures. Gamma cells are the most commonly used emitters for plant mutation induction, as of 2004 there were about 200 gammacells in use world–wide (IAEA, 2004). However, the gamma radiation source has a distinct advantage for prolonged treatments in that it may be placed in a controlled environment chamber.

Ultraviolet light:

Ultraviolet light or UV light is a non-ionizing radiation at the wavelength commonly employed (e.g. the 2537 nm line of mercury germicidal lamps), but it will be included in this discussion because it has frequently been used in plant mutation induction especially in pollen grains, cell and/or plant tissue cultures. UV radiations are generally divided into three classes: UV-A, UV-B and UV-C. The UV-C region of the UV spectrum includes wavelengths below 280 nm; UV radiation in the UV-B region those from 280 to 320 nm, and UV wavelengths from 320 to 390 nm make up the UV-A region of the spectrum. Ultraviolet light has limited tissue penetration and its use is restricted to treating sensitive materials, often single cells or single layer tissues, such as spores, suspension cell cultures and pollen grains. However, the increasing use of cell and tissue culture for mutation breeding of plants has led to increased use of UV light as a mutagenic agent, especially when single mutant genes are sought (see Chapter 8). In order to make quantitative assessments of experimental results, it proved necessary to use monochromatic (or near monochromatic) UV-C light because it has confirmed biological effects on photosynthesis, dark respiration and transpiration (Castronuovo et al., 2014).

Bulgaria: Mutant durum varieties have occupied about 90% of the cultivating area since the 1980's. China: Each of the following mutant varieties has cumulatively been grown on acreage of more than 10 million ha: rice varieties Yuanfengzao, Zhefu 802 and Yangdao No. 6; wheat variety Yangmai 156; and the cotton variety Lumian no.1.

Costa Rica: Rice variety Camago occupied 30% of the cultivated area.

Europe: Many barley varieties widely grown in Europe are derived from mutant varieties Diamant or Golden Promise.

India: Mutant varieties are the prevailing varieties for pulses and legumes, for example the TAU-1 mutant of blackgram has occupied 95% of the blackgram acreage in the State of Maharashtra and the groundnut varieties of the TG series (e.g. TG24 and TG37) cover 40% of the groundnut acreage.

Italy: Durum wheat cultivation area was significantly expanded due to the cold tolerant mutant varieties (e.g. Creso).

Japan: Most rice varieties carry the sd1 mutant allele from the variety Reimei. Japanese pear cultivation was rescued from extinction by the development of disease resistant mutant varieties, Gold Nijisseiki and its derivatives.

Pakistan: The wheat mutant variety Kiran 95 and the cotton mutant variety NIAB-78 were planted on over 30% and 80% of the cultivation area for each crop, respectively.

USA: The rice mutant variety Calrose 76 was the sd1 donor for more than 10 successful varieties. Star Ruby and Rio Red are the two most important commercial grapefruit varieties (with the trade mark Rio Star).

Vietnam: VND and DT serial mutant rice varieties (e.g. VND95-20 and DT38) have been cultivated on more than half million ha per year during the last decade, and DT serial mutant soybean varieties have been cultivated on more than 50% area with DT84 being the leading variety in the past 10 years.

POLYPLOIDY

Polyploids are organisms with multiple sets of chromosomes in excess of the diploid number (Acquaah, 2007; Chen, 2010; Comai, 2005; Ramsey and Schemske, 1998). Polyploidy is common in nature and provides a major mechanism for adaptation and speciation. Approximately 50-70% of angiosperms, which include many crop plants, have undergone polyploidy during their evolutionary process (Chen et al., 2007). Flowering plants form polyploids at a significantly high frequency of 1 in every 100,000 plants (Comai, 2005). Many studies have been carried out to understand the nature of polyploidism. This chapter seeks to illuminate some of these studies and explain the applications and implications of polyploidy in plant breeding and other commercial ventures. To understand polyploidy, a few basic notations need be defined. The basic complete set of chromosomes is designated by "x" while the total number of chromosomes in a somatic cell is designated "2n". The total number of chromosomes in a somatic cell is twice the haploid number (n) in the gametes (Acquaah, 2007; Otto and Whitton, 2000).

Classification of Polyploids

Polyploids may be classified based on their chromosomal composition into either **euploids** or **aneuploids**. Euploids constitute the majority of polyploids.

Euploidy

Euploids are polyploids with multiples of the complete set of chromosomes specific to a species. Depending on the composition of the genome, euploids can be further classified into either **autopolyploids** or **allopolyploids**. Tetraploidy is the most common class of euploids (Comai, 2005).

Unit II

Genetic and Molecular basis

Autopolyploidy

Autopolyploids are also referred to as autoploids. They contain multiple copies of the basic set (x) of chromosomes of the same genome (Acquaah, 2007; Chen, 2010). Autoploids occur in nature through union of unreduced gametes and at times can be artificially induced (Chen, 2010).

Natural autoploids include tetraploid crops such as alfafa, peanut, potato and coffee and triploid bananas. They occur spontaneously through the process of chromosome doubling. Chromosome doubling in autoploids has varying effect based on the species. Spontaneous chromosome doubling in ornamentals and forage grasses has led to increased vigour. For instance, ornamentals such as tulip and hyacinth, and forage grasses such as ryegrasses have yielded superior varieties following spontaneous chromosome doubling (Acquaah, 2007). Due to the observed advantages in nature, breeders have harnessed the process of chromosome doubling *in vitro* through induced polyploidy to produce superior crops. For example, induced autotetraploids in the watermelon crop are used for the production of seedless triploid hybrids fruits (Fig 5.1) (Wehner, 2008). Such polyploids are induced through the treatment of diploids with mitotic inhibitors such as dinitroaniles and colchicine (Compton et al., 1996). To determine the ploidy status of induced polyploids, several approaches may be used. These include, chloroplast count in guard cells, morphological features such as leaf, flower or pollen size (gigas effect) and flow cytometry (Brummer et al., 1999; Heping et al., 2008).

Allopolyploidy

Allopolyploids are also called alloploids. They are a combination of genomes from different species (Acquaah, 2007). They result from hybridization of two or more genomes followed by chromosome doubling or by the fusion of unreduced gametes between species (Acquaah, 2007; Chen, 2010; Jones et al., 2008; Ramsey and Schemske, 1998). This process is key in the process of speciation for angiosperms and ferns (Chen, 2010) and occurs often in nature. Economically important natural alloploid crops include strawberry, wheat, oat, upland cotton, oilseed rape, blueberry and mustard (Acquaah, 2007; Chen, 2010). To differentiate between the sources of the genomes in an alloploid, each genome is designated by a different letter. For example, the origin of the cultivated mustards (*Brassica spp*) has been well explained by Nagaharu in the triangle of U with each species represented by a distinct letter (Fig 5.2) (Bellostas et al., 2007; Nelson et al., 2009).

The hybridized genomes differ in their degree of homology with some being able to pair during mitosis and/or meiosis while others not. When only segments of the chromosomes of the combining genomes differ, the phenomenon is called segmental alloploidy. These chromosomes are similar but not homologous and are called homeologous chromosomes.

Such chromosomes indicate ancestral homology (Acquaah, 2007). Induced alloploidy is not common. However, it has been used in some Genus such as *Cucumis* to elucidate the molecular mechanisms involved in diploidization (tendency of polyploids to act as diploids) (Chen et al., 2007). In this study, an allotetraploid was induced by hybridization between *Cucumis sativus* and *Cucumis hystrix* followed by chromosome doubling. Cytogenetic studies were carried out in the following generations to establish the molecular mechanisms involved stabilization of newly formed allopolyploids which include neo-functionalization and sub-functionalization (Chen et al., 2007; Comai, 2005).

Aneuploidy

Aneuploids are polyploids that contain either an addition or subtraction of one or more specific chromosome(s) to the total number of chromosomes that usually make up the ploidy of a species (Acquaah, 2007; Ramsey and Schemske, 1998). Aneuploids result from the formation of univalents and multivalents during meiosis of euploids (Acquaah, 2007). For example, several studies have found that 30-40% of progeny derived from autotetraploid maize are aneuploids (Comai, 2005). With no mechanism of dividing univalents equally among daughter cells during anaphase I, some cells inherit more genetic material than others (Ramsey and Schemske, 1998). Similarly, multivalents such as homologous chromosomes may fail to separate during meiosis leading to unequal migration of chromosomes to opposite poles. This mechanism is called non-disjunction (Acquaah, 2007). These meiotic aberrances result in plants with reduced vigor.

Mechanisms of Polyploidy Formation

Several cytological mechanisms are known to spontaneously induce polyploidy in plants (Ramsey and Schemske, 1998). One such route involves non-reduction of gametes during meiosis a process called meiotic nuclear restitution. The formed gametes (2n) contain the somatic nuclear condition of cells. Meiotic aberrations related to spindle formation, spindle function and cytokinesis have been implicated in this process (Ramsey and Schemske, 1998). The subsequent union of reduced and non-reduced gametes leads to the formation of polyploids (Acquaah, 2007; Ramsey and Schemske, 1998). For example, autotetraploids may be formed in a diploid population through the union of two unreduced 2n gametes as was found in the F1 progenies of open-pollinated diploid apples (Ramsey and Schemske, 1998). Similarly, spontaneous allotetraploids were formed in 90% of F2 progenies of interspecific crosses between *Digitalis ambigua* and *Digitalis purpurea*, which are common ornamental plants (Ramsey and Schemske, 1998). Another example is the formation of autohexaploid *Beta vulgaris* (sugar beet) and alfalfa from cultivated autotetraploid varieties apparently from the union of reduced (2x) and unreduced (4x) gametes (Bingham, 1968; Hornsey, 1973).

Another major route for polyploid formation is through somatic doubling of chromosomes during mitosis. In nature, the formation of polyploids as a result of mitotic aberrations has been reported in the meristematic tissue of several plant species including tomato and in non-meristematic tissues of plants such as bean (Coleman, 1950; Ramsey and Schemske, 1998). Artificial inducement of polyploids through the inhibition of mitosis is routine in plant breeding. High temperatures above 40°C have been used to induce tetraploid and octoploid corn seedlings albeit with low success of 1.8% and 0.8% respectively (Randolph, 1932). Currently, chemical mitotic inhibitory agents such as colchicine or dinitroanilines are used to induce polyploidy in crop plants. A typical example is the production of tetraploid watermelon plants for the production of seedless triploid watermelon (Compton et al., 1996).

In addition, an uncommon mechanism of polyploid formation involves polyspermy where one egg is fertilized by several male nucleuses as commonly observed in orchids (Ramsey and Schemske, 1998).

Effect of polyploidy on inheritance and population genetics

An immediate consequence of polyploidy is the change in gametic and filial frequencies (Comai, 2005). This is because polyploids have multiple alleles associated with a single locus. For example, a hexaploid has six alleles per locus while a tetraploid has four. The genetics of polyploids is often complicated by multi-allelism at loci thus altering segregation ratios and inheritance patterns expected in diploids. Provided a polyploid species behaves like a diploid at meiosis through normal bivalent pairing (disomic inheritance), such as in wheat or tobacco, normal biometric analysis of inheritance apply (Kearsey and Pooni, 1998). However, several autotetraploid crop plants including potatoes, coffee and lucerne and some forage grasses have tetrasomic inheritance (Killick, 1971). With this knowledge, it is necessary to make accommodations in population structure and breeding strategy to account for differences in gamete structure (Katepa-Mupondwa et al., 2002). For example, breeding schemes that maximize heterozygosity are frequently used for the autotetraploid alfalfa in an attempt to utilize multi-allelic interactions (Katepa-Mupondwa et al., 2002). Altered genotypic ratios are apparent in polyploids when compared with diploids. For example an arbitrary locus with B (dominant) and b (recessive) alleles, following selfing, an autotetraploid (*BBbb*) would produce 5 possible genotypes while a diploid (*Bb*) would generate 3 possible genotypes (Fig 5.5) (Acquaah, 2007; Killick, 1971). Distinguishing a quadruplex (*BBBB*) from a triplex (*BBBb*) in the segregating population using a progeny test presents difficulty in breeding because both would breed true to the dominant allele. An extra generation would be required to identify the triplex by observing the formation of duplex plants (Acquaah, 2007).

Effect of polyploidy on sterility

Since autoploids contain more than two homologous chromosomes, meiosis results in the formation of univalents and multivalent, unlike in diploids where bivalents are usually formed (Acquaah, 2007). For instance during meiosis, autotetraploids may form bivalents, quadrivalents and univalents (Fig 5.6). The ratio of these gametes following meiosis determines the fertility of a polyploid individual. Univalents and trivalents result in non-functional sterile gametes and are the most common in triploids, making them sterile.

Rigorous and effective selection strategies for fertile autoploids are practiced in the development of inbred lines. Breeders rogue out autoploids with low seed set as well as those with morphological abnormalities (Andrus et al., 1971). Sterile alloploids arise from the pairing of homeologous chromosomes from separate genomes during meiosis instead of homologous chromosome (Chen et al., 2007; Levi et al., 2002). This results in non-functional gametes. A viable allopolyploid requires a diploid-like meiosis behavior to establish disomic inheritance and full fertility. Fertility problems in allopolyploids also occur when crossing crops of different ploidy levels as a result of formation of multivalents. To improve fertility, breeders use the parent with the lowest chromosome number as the female parent so as to increase seed set (Olmo, 1952).

Common Applications of Ploidy in Crop Plants

Mutation breeding

High frequencies of chromosome mutations are desirable in modern breeding techniques, such as tilling, as they provide new sources of variation. The multiallelic nature of loci in polyploids has many advantages that are useful in breeding. The masking of deleterious alleles, that may arise from induced mutation, by their dominant forms cushions polyploids from lethal conditions often associated with inbred diploid crops (Gaul, 1958). This concept has been instrumental in the evolution of polyploids during bottlenecks where there is enforced inbreeding (Comai, 2005). Mutation breeding exploits the concept of gene redundancy and mutation tolerance in polyploid crop improvement in two ways. First, polyploids are able to tolerate deleterious allele modifications post-mutation, and secondly, they have increased mutation frequency because of their large genomes resulting from duplicated condition of their genes (Gaul, 1958). The high mutation frequencies observed with polyploids may be exploited when trying to induce mutations in diploid cultivars that do not produce enough genetic variation after a mutagenic treatment. This approach has been used in mutation breeding of *Achimenes sp.* (nut orchids) by first forming autotetraploids through colchicine treatment followed by the application of fast neutrons and X-rays. In this

study, the autotetraploids were found to have 20-40 times higher mutation frequency than the corresponding diploid cultivar due to the large genome (Broertjes, 1976).

Seedless fruits

The seedless trait of triploids has been desirable especially in fruits. Commercial use of triploid fruits can be found in crops such as watermelons and are produced artificially by first developing tetraploids which are then crossed with diploid watermelon. In order to set fruit, the triploid watermelon is crossed with a desirable diploid pollen donor.

Bridge crossing

Another breeding strategy that utilizes the reproductive superiority of polyploids is bridge crossing. When sexual incompatibilities between two species are due to ploidy levels, transitional crosses can be carried out followed by chromosome doubling to produce fertile bridge hybrids. This method has been used to breed for superior tall fescue grass (*F. arundinacea*) from Italian ryegrass (2n=2x=14) and tall fescue (2n=6x=42) by using meadow grass (*Fescue pratensis*) as a bridge species (Fig.5.7) (Acquaah, 2007). The same principle has been applied in fixing heterozygosity in hybrids by doubling the chromosomes in the superior progeny (Comai, 2005).

Class: I M.Sc. BT Course Name: Agricultural Biotechnology
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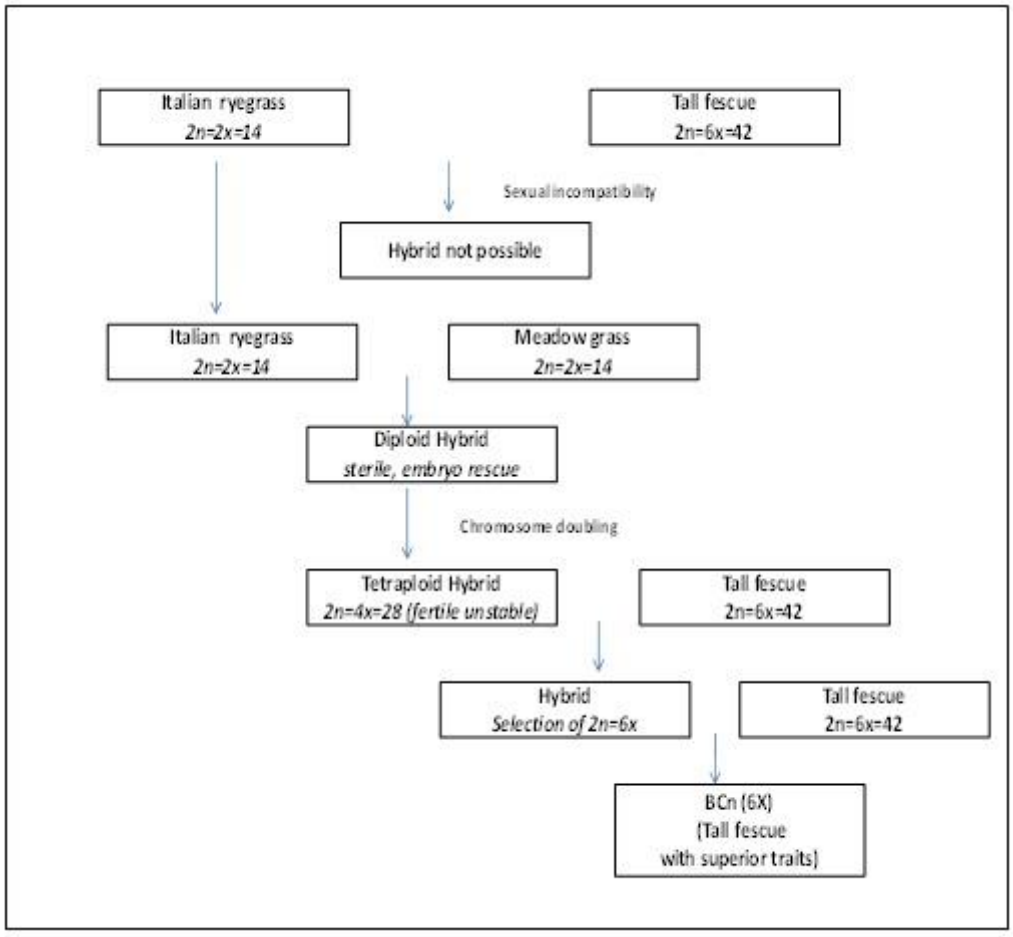
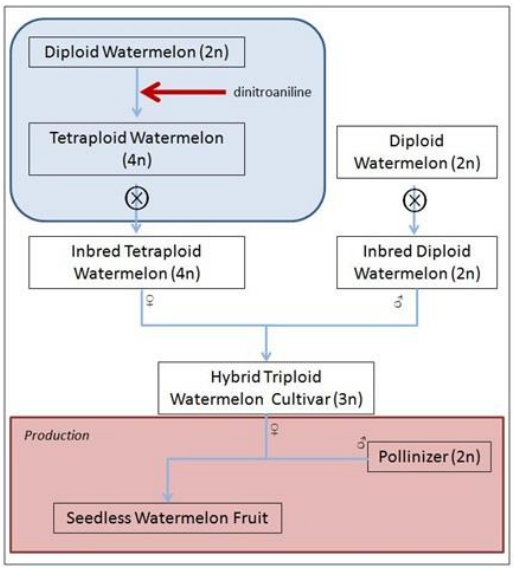


Figure 5.7 The development of superior tall fescue grass through bridge crossing and induced tetraploidy.



Ornamental and forage breeding

One of the immediate and obvious consequences of polyploidy in plants is an increase in cell size which in turn leads to enlarged plant organs, a phenomenon termed gigas effect (Fig 5.4) (Acquaah, 2007; Levin, 1983; Stebbins, 1971). For example, the volume of tetraploid cells usually is about twice that of their diploid progenitors (Acquaah, 2007; Emsweller and Ruttle, 1941; Levin, 1983; Schepper et al., 2001). The increase in cell volume however is mainly attributed to increased water and not biomass. Therefore, its application is limited for breeding agronomically important crops such as cereals. Although chromosome doubling may result in significantly larger seeds and increased seed-protein content in cereal crops, this advantage is offset by low seed set (Dhawan and Lavania, 1996). In contrast, the gigas effect has been explored in tree, ornamental, forage crop and fruit breeding (Emsweller and Ruttle, 1941; Schepper et al., 2001). For example, through induced polyploidy, breeders have developed Bouschet tetraploid grapes with more yield and juice content than the diploid progenitor Alicante (Olmo, 1952). Ornamental crops such as snapdragons and marigolds have been bred through chromosome doubling to improve the quality and size of their blossoms (Emsweller and Ruttle, 1941). A strong inverse correlation between DNA content and development rates in plants has been reported by several authors (Levin, 1983; Smith and Bennett, 1975). It has been attributed to lower auxin levels, reduced surface to volume ratio and altered nuclear surface to cell volume ratio (Acquaah, 2007; Levin, 1983). The slower growth rate of polyploids allows them to flower later and for a longer period of time than their diploid progenitors (Levin, 1983). This quality may be of interest especially in ornamental breeding.

Production of apomictic crops

Apomixis provides another avenue for use of polyploids in breeding. Apomixis provides an avenue for the production of seeds asexually through parthenogenesis. Most apomictic plants are polyploid but most polyploid plants are not apomictic (Otto and Whitton, 2000). In plants capable of both sexual and asexual reproduction, polyploidy promotes the latter (Dhawan and Lavania, 1996; Levin, 1983). Obligate apomicts are the most desired of hybrids but little gain has been realized towards their development. However, it has been suggested that obligate apomicts may be induced through development of very high ploidy plants (Levin, 1983). An example of an obligate apomict achieved at high ploidy level is the octoploid of the grass, *Themeda triandra* (Levin, 1983).

Disease resistance through aneuploidy

Aneuploidy has been applied in breeding to develop disease resistant plants through the addition of an extra chromosome into the progeny genome. An example is the transfer of leaf

rust resistance to *Tricum aestivum* from *Aegilops umbellulata* through backcrossing. In addition, other breeding strategies utilizing aneuploidy have been explored including chromosome deletion, chromosome substitution and supernumerary chromosomes (Acquaah, 2007).

Industrial applications of polyploidy

Chromosome doubling is reported to have an apparent effect on many physiological properties of a plant. The most discernable of these has been the increase in secondary as well as primary metabolism (Levin, 1983). The resulting increase in secondary metabolites, in some cases by 100%, after chromosome doubling has been widely exploited in the breeding of narcotic plants such as *Cannabis, Datura* and *Atropa* (De Jesus-Gonzalez and Weathers, 2003; Dhawan and Lavania, 1996; Levin, 1983). *In vitro* secondary metabolite production systems that exploit polyploidism have also been developed. The production of the antimalarial sesquiterpene artemisinin has been enhanced six fold by inducing tetraploids of the wild diploid *Artemisia annua* L. (clone YUT16) (De Jesus-Gonzalez and Weathers, 2003). In addition, commercial synthesis of sex hormones and corticosteroids has been improved significantly by artificial induction of tetraploids from diploid *Dioscorea zingiberensis*, native to China (Heping et al., 2008). Attempts have been made to improve the production of pyrethrin, a botanical insecticide, by chromosome doubling of *Chrysanthemum cinerariifolium* (Liu and Gao, 2007). Other plants whose production of terpenes has increased following artificial chromosome doubling include *Carum cari, Ocimum kilmandscharicum* and *Mentha arvensis* (Bose and Choudhury, 1962; Levin, 1983). The enhanced production of secondary metabolites such as alkaloids and terpenes in polyploids may concurrently offer resistance to pests and pathogens. Experiments with diploid *Glycine tabacina*, a forage legume, and its tetraploid forms to measure resistance to leaf rust, *Phakopsora pachyrhizi*, established that 42% of the tetraploid plants were resistant compared to 14% of the diploid plants (Levin, 1983). Similar results were observed while comparing resistance to insects and the clover eel nematode between *Trifolium pratense* (red clover) tetraploids and diploids (Mehta and Swaminathan, 1957).

Molecular Markers:

A molecular marker is a DNA sequence in the genome which can be located and identified. As a result of genetic alterations (mutations, insertions, deletions), the base composition at a particular location of the genome may be different in different plants.

These differences, collectively called as polymorphisms can be mapped and identified. Plant breeders always prefer to detect the gene as the molecular marker, although this is not always

possible. The alternative is to have markers which are closely associated with genes and inherited together.

The molecular markers are highly reliable and advantageous in plant breeding programmes:

i. Molecular markers provide a true representations of the genetic makeup at the DNA level.

ii. They are consistent and not affected by environmental factors.

iii. Molecular markers can be detected much before development of plants occur.

iv. A large number of markers can be generated as per the needs.

Basic principle of molecular marker detection:

Let us assume that there are two plants of the same species—one with disease sensitivity and the other with disease resistance. If there is DNA marker that can identify these two alleles, then the genome can be extracted, digested by restriction enzymes, and separated by gel electrophoresis. The DNA fragments can be detected by their separation.For instance, the disease resistant plant may have a shorter DNA fragment while the disease — sensitive plant may have a longer DNA fragment.

Molecular markers are of two types:

1. Based on nucleic acid (DNA) hybridization (non-PCR based approaches).

2. Based on PCR amplification (PCR-based approaches).

Markers Based On DNA Hybridization:

The DNA piece can be cloned, and allowed to hybridize with the genomic DNA which can be detected. Marker-based DNA hybridization is widely used. The major limitation of this approach is that it requires large quantities of DNA and the use of radioactivity (labeled probes).

Restriction fragment length polymorphism (RFLP):

RFLP was the very first technology employed for the detection of polymorphism, based on the DNA sequence differences. RFLP is mainly based on the altered restriction enzyme sites, as a result of mutations and re-combinations of genomic DNA. An outline of the RFLP analysis is given in Fig. 53.2, and schematically depicted in Fig. 53.3.The procedure basically involves the isolation of genomic DNA, its digestion by restriction enzymes, separation by

electrophoresis, and finally hybridization by incubating with cloned and labeled probes (Fig. 53.2).

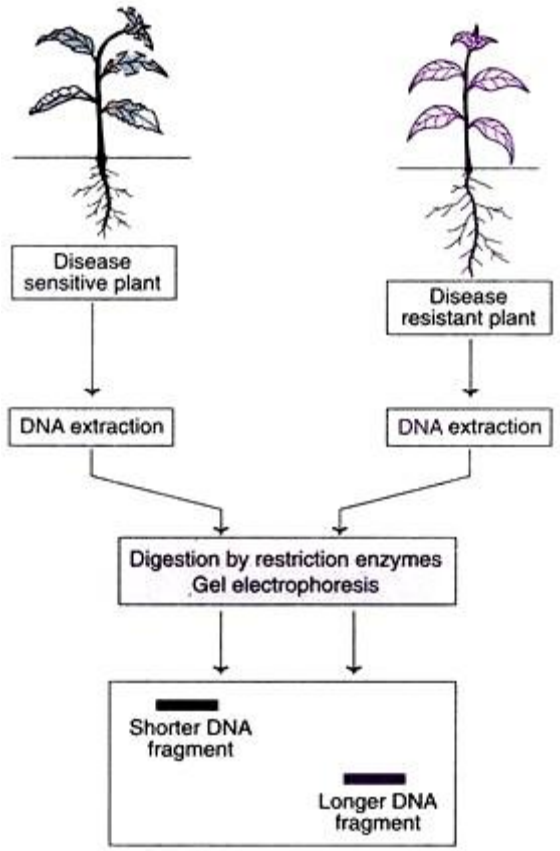


***Fig. 53.1** : Basic principle of molecular marker detection (screening of genotypes for the identification of DNA markers).*

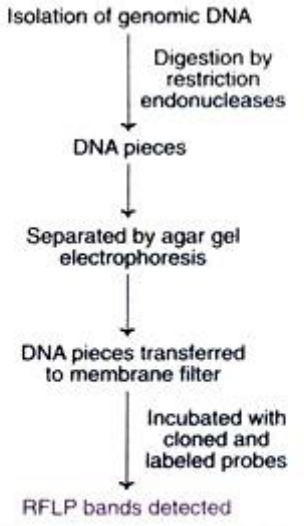


Fig. 53.2 : An outline of restriction fragment length polymorphism (RFLP) analysis as a molecular marker in plant breeding.

Based on the presence of restriction sites, DNA fragments of different lengths can be generated by using different restriction enzymes. In the Fig. 53.3, two DNA molecules from two plants (A and B) are shown. In plant A, a mutations has occurred leading to the loss of restriction site that can be digested by EcoRI.

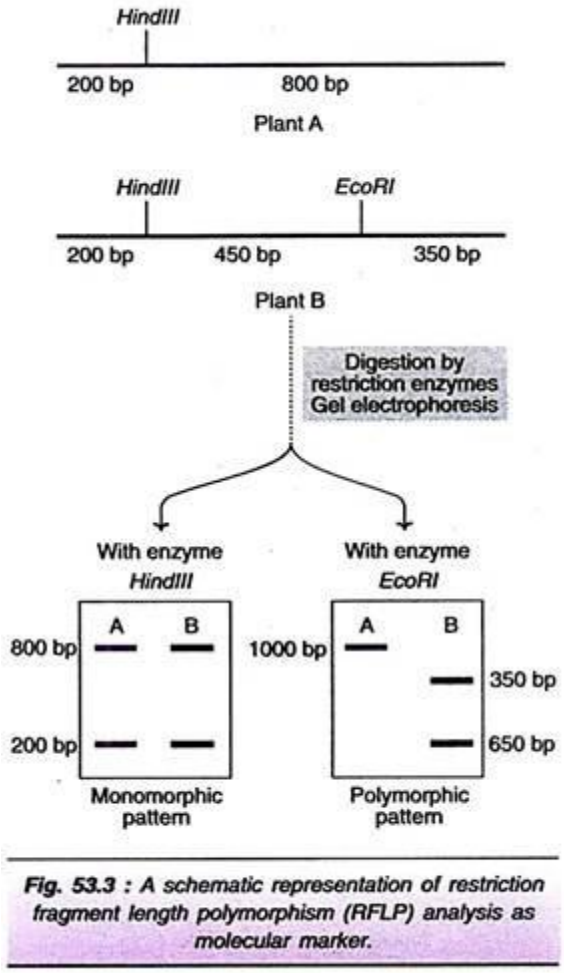


Fig. 53.3 : A schematic representation of restriction fragment length polymorphism (RFLP) analysis as molecular marker.

The result is that when the DNA molecules are digested by the enzyme Hindlll, there is no difference in the DNA fragments separated. However, with the enzyme EcoRI, plant A DNA molecules is not digested while plant B DNA molecule is digested. This results in a polymorphic pattern of separation.

Markers Based on PCR Amplification:

Polymerase chain reaction (PCR) is a novel technique for the amplification of selected regions of DNA .The advantage with PCR is that even a minute quantity of DNA can be amplified. Thus, PCR-based molecular markers require only a small quantity of DNA to start with.

PCR-based markers may be divided into two types:

1. Locus non-specific markers e.g. random amplified polymorphic DNA (RAPD); amplified fragment length polymorphism (AFLP).

2. Locus specific markers e.g. simple sequence repeats (SSR); single nucleotide polymorphism (SNP).

Random amplified polymorphic DNA (RAPD) markers:

RAPD is a molecular marker based on PCR amplification. An outline of RAPD is depicted in Fig. 53.4. The DNA isolated from the genome is denatured the template molecules are annealed with primers, and amplified by PCR.

Single short oligonucleotide primers (usually a 10-base primer) can be arbitrarily selected and used for the amplification DNA segments of the genome (which may be in distributed throughout the genome). The amplified products are separated on electrophoresis and identified.

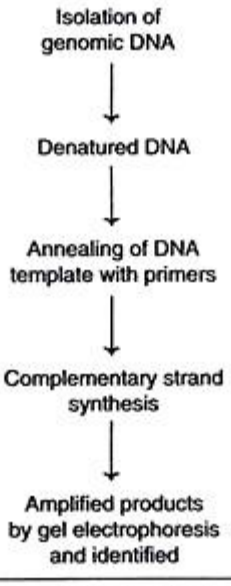


Fig. 53.4 : An outline of random amplified polymorphic DNA (RAPD) analysis as a molecular marker in plant breeding.

Based on the nucleotide alterations in the genome, the polymorphisms of amplified DNA sequences differ which can be identified as bends on gel electrophoresis. Genomic DNA from two different plants often results in different amplification patterns i.e. RAPDs. This is based on the fact that a particular fragment of DNA may be generated from one individual, and not from others. This represents polymorphism and can be used as a molecular marker of a particular species.

Amplified fragment length polymorphism (AFLP):

AFLP is a novel technique involving a combination of RFLP and RAPD. AFLP is based on the principle of generation of DNA fragments using restriction enzymes and oligonucleotide

adaptors (or linkers), and their amplification by PCR. Thus, this technique combines the usefulness of restriction digestion and PCR.

The DNA of the genome is extracted. It is subjected to restriction digestion by two enzymes (a rare cutter e.g. MseI; a frequent cutter e.g. EcoRI). The cut ends on both sides are then ligated to known sequences of oligonucleotides (Fig. 53.5).

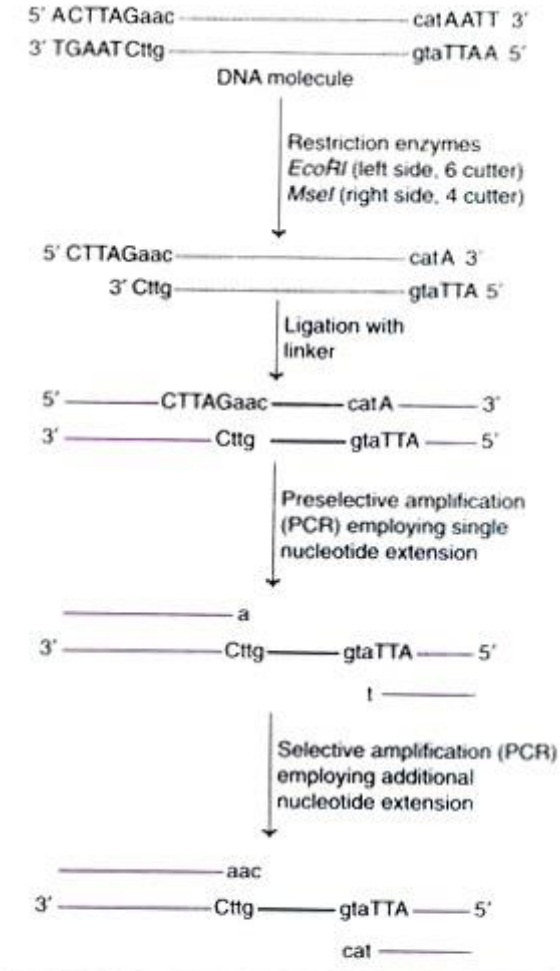


***Fig. 53.5** : A diagrammatic representation of the amplified fragment length polymorphism (AFLP) (**Note** : The lower case letters represent the sequences found within the amplified region; the coloured lines indicate linkers).*

PCR is now performed for the pre-selection of a fragment of DNA which has a single specific nucleotide. By this approach of pre-selective amplification, the pool of fragments can be reduced from the original mixture. In the second round of amplification by PCR, three nucleotide sequences are amplified.

This further reduces the pool of DNA fragments to a manageable level (< 100). Autoradiography can be performed for the detection of DNA fragments. Use of radiolabeled primers and fluorescently labeled fragments quickens AFLP.

AFLP analysis is tedious and requires the involvement of skilled technical personnel. Hence some people are not in favour of this technique. In recent years, commercial kits are made available for AFLP analysis. AFLP is very sensitive and reproducible. It does not require prior knowledge of sequence information. By AFLP, a large number of polymorphic bands can be produced and detected.

Sequence tagged sites (STS):

Sequence tagged sites represent unique simple copy segments of genomes, whose DNA sequences are known, and which can be amplified by using PCR. STS markers are based on the polymorphism of simple nucleotide repeats e.g. $(GA)_n$, $(GT)_n$, $(CAA)_n$ etc. on the genome. STS have been recently developed in plants. When the STS loci contain simple sequence length polymorphisms (SSLPs), they are highly valuable as molecular markers. STS loci have been analysed and studied in a number of plant species.

Microsatellites:

Microsatellites are the tandemly repeated multi-copies of mono-, di-, tri- and tetra nucleotide motifs. In some instances, the flanking sequence of the repeat sequences may be unique. Primers can be designed for such flanking sequences to detect the sequence tagged microsatellites (STMS). This can be done by PCR.

Sequence characterized amplified regions (SCARs):

SCARs are the modified forms of STS markers. They are developed by PCR primer that are made for the ends of RAPD fragment. The STS-converted RAPD markers are sometimes referred to as SCARs. SCARs are useful for the rapid development of STS markers.

Molecular Marker Assisted Selection:

Selection of the desired traits and improvement of crops has been a part of the conventional breeding programmes. This is predominantly based on the identification of phenotypes. It is now an accepted fact that the phenotypes do not necessarily represent the genotypes. Many a times the environment may mark the genotype. Thus, the plant's genetic potential is not truly reflected in the phenotypic expression for various reasons.

The molecular marker assisted selection is based on the identification of DNA markers that link/ represent the plant traits. These traits include resistance to pathogens and insects,

tolerance to abiotic stresses, and various other qualitative and quantitative traits. The advantage with a molecular marker is that a plant breeder can select a suitable marker for the desired trait which can be detected well in advance. Accordingly, breeding programmes can be planned.

The following are the major requirements for the molecular marked selection in plant breeding:

i. The marker should be closely linked with the desired trait.

ii. The marker screening methods must be efficient, reproducible and easy to carry out.

iii. The analysis should be economical.

Molecular Breeding:

With rapid progress in molecular biology and genetic engineering, there is now a possibility of improving the crop plants with respect to yield and quality. The term molecular breeding is frequently used to represent the breeding methods that are coupled with genetic engineering techniques.

Improved agriculture to meet the food demands of the world is a high priority area. For several years, the conventional plant breeding programmes (although time consuming) have certainly helped to improve grain yield and cereal production.

The development of dwarf and semi-dwarf varieties of rice and wheat have been responsible for the 'Green Revolution', which has helped to feed millions of poverty-stricken people around the world. Many developments on the agriculture front are expected in the coming years as a result of molecular breeding.

Linkage analysis:

Linkage analysis basically deals with studies to correlate the link between the molecular marker and a desired trait. This is an important aspect of molecular breeding programmes. Linkage analysis has to be carried out among the populations of several generations to establish the appropriate linkage. In the earlier years, linkage analysis was carried out by use of isoenzymes and the associated polymorphisms. Molecular markers are now being used. The techniques employed for this purpose have already been described.

Unit II
Genetic and Molecular basis

Quantitative Trait Loci:

These are many characteristics controlled by several genes in a complex manner. Some good examples are growth habit, yield, adaptability to environment, and disease resistance. These are referred to as quantitative traits. The locations on the chromosomes for these genes are regarded as quantitative trait loci (QTL).

The major problem, the plant breeder faces is how to improve the a complex character controlled by many genes. It is not an easy job to manipulate multiple genes in genetic engineering. Therefore, it is a very difficult and time consuming process. For instance, development of Golden Rice (with enriched pro-vitamin A) involving the insertion of just three genes took about seven years.

Arid and Semi-Arid Plant Biotechnology:

The terms arid zone is used to refer to harsh environmental conditions with extreme heat and cold. The fields have limited water and minerals. It is different task to grow plants and achieve good crop yield in arid zones. Semi-arid regions are characterized by unpredictable weather, inconsistent rainfall, long dry seasons, and poor nutrients in the soil.

Most parts of India and many other developing countries (Africa, Latin America, and Southeast Asia) have semi- arid regions. Crops like sorghum, millet, groundnut and cowpea are mostly grown in semi-arid tropics. Besides unpredictable weather, biotic and abiotic stresses contribute to crop loss in these areas.

The biotechnological approaches for the breeding programmes in the semi-arid regions should cover the following areas:

i. Development of crops that are tolerant to drought and salinity.

ii. Improvements to withstand various biotic and abiotic stresses.

iii. Micro-propagation techniques to spread economically important plants which can withstand harsh environmental conditions.

Some success has been achieved in improving sorghum, millet and legume crops that are grown in semi-arid regions. Genetic transformation in sorghum was possible by using micro projectile method.

Unit II

Genetic and Molecular basis

Greenhouse and Green-home Technology:

Greenhouse literally means a building made up of glass to grow plants. Green houses are required to grow regenerated plants for further propagation and for growing plants to maturity. Greenhouses are the intermediary stages involving the transitional step between the plant cultures and plant fields. The purpose of greenhouses is to acclimatize and test the plants before they are released into the natural environment.

The plants are grown in greenhouse to develop adequate root systems and leaves so as to withstand the field environment. The greenhouses are normally equipped with cooling systems to control temperature. Greenhouses have chambers fitted with artificial lights. It is possible to subject the plants to different lighting profiles. In recent years many improvements have been made in the development of more suitable greenhouses. These include the parameters such as soil, and humidity.

The major limitation of greenhouse technology is an increase in CO_2 production that in turn increases temperature. Some approaches are available to control temperature. Green home technology is a recent development. In this case, temperature is controlled by using minimum energy.

The Origin & History of Agriculture

From earliest times human distributions have been correlated with the distribution of plants. The history and development of agriculture is intimately related to the development of civilization. For last 30-40,000 yrs (advent of cromagnon) very little physical evolution is evident in fossil record but there has been tremendous cultural evolution. The advent of stationary human societies and consequent development of civilization were possible only after the establishment of agriculture. Humans did not “put down roots” and remain in one place until they learned to cultivate the land and collect and store agricultural crops. The origin of agriculture provided “release time” for the development of art, writing, culture and technology. Hunter Gatherers The earliest humans lived in small bands of several families (up to 50 or so). For over a million years (paleolithic or old stone age) humans obtained food by hunting wild animals and gathering plants. They depended almost completely on the local environment for their sustenance. Such hunter gathering societies existed extensively until 10,000 yrs ago. A few isolated groups continue to this day. Paleolithic cultures were nomadic by necessity. They wandered as small family groups in search of game and edible plants. Meat was their primary source of protein, sugars & many vitamins were provided by fruits & berries, starches from roots and seed, and oils and vitamins from nuts. As seasons changed, nomadic peoples moved on to follow game, gathering whatever plants were available as they

traveled. The Beginnings of Agriculture Agriculture seems to have arisen in temperate regions before it showed up in the tropics; food was available year round in the tropics with less need to migrate with the game. Around 10-12,000 years ago agriculture originated at least 4 separate times in 4 different places: Mideast, fertile crescent China, Yellow River Egypt, Aswan, Central America, Tehuacan valley.

A possible scenario:

1. Nomadic tribes migrated annually in the fall and spring from the foothills of surrounding mountains to nearby valleys following the natural migrations of animal herds. Also, valleys provided the plants needed for food, fiber and fuel during the winter that were not available at higher elevations. In the spring the animals moved back to the foothills and spent summer in cooler locations
2. This cycle was repeated each year. They probably used same routes and same camps each season
3. Each camp had a designated trash heap orcompost pile; seeds fruits wastes were thrown there.
4. Plants sprouted in these rich compost beds. As people returned year after year must have noticed and took advantage of fruits and vegetables growing in these beds.
5. The realization of choice plants growing near camp could have led to experimental "farming". With more and more successes they could have cultivated more and more plants.
6. They became increasingly dependent on such activities. Staying in one place also meant fewer hazards, more leisure time, greater population size and a much more sedentary lifestyle.
7. Such sedentary lifestyle would have promoted other important changes: the accumulation of material goods, a division of labor, not everyone needed to be farmers, people became specialists as potters, weavers, tanners, artisans and scholars
8. Biological evolution was supersceded by "cultural" evolution; advanced civilizations rapidly evolved Earliest Agriculture The first plants to be cultivated were probably the ones that had been originally gathered by the nomadic tribes, were locally abundant, were easily cultivated, and had other uses besides just as food. In the Near East barley may have been the 1st crop domesticated there. Soon followed by wheat, barley, peas, lentils, vetch. At the same time dogs, goats, and sheep were domesticated.

In China rice, millet, rape , and hemp may have been some of the first crops, as cattle, pigs, dogs, and poultry were domesticated. In the Tehuacan Valley corn probably the first cultivated plant. Later squash, avacado, gourds, beans, and chili peppers were grown while

dogs and later turkeys were domesticated. We do know that every important civilization depended on cereal crops (grasses such as; rice, corn, wheat, grains) as mainstay of their agricultural base: eg. millets (Setaria, Echinochloa, Panicum) were among the earliest grains to be cultivated; eg. in China & SE Asia, rice (Oryza) was widely cultivated; eg. in Middle East, wheat (Triticum) was cultivated in hilly regions; eg. barley (Hordeum) was grown in southern Egypt; eg. Maize (Zea) was the main cereal crop in the Americas. Cereal Grains: The use of wild grass grains and development of more productive strains were central to further development of settled villages and great civilizations. As food plants they have several beneficial traits: a. high yield: a large amount of grain/acre under cultivation; each grain is rich in carbohydrates, minerals, fats, vitamins & proteins b. the grains were compact and dry and highly suitalb for long term storage c. grains could be easily ground into flour which could be used in a variety of different ways d. the stems and leaves (straw) could be woven or thatched into baskets, bedding and housing e. cereal plants easily reproduce by runners increasing yields even more f. domesticated animals grazing in these grains would not only have provided meat and hides but would have increased their yield by spreading seeds and fertilizing the crops.

Early Root and Stem Crops: Many plants with large edible roots and stems were easily cultivated and harvested. They tended to have high levels of nutritious starch and could be easily replanted using leftover pieces of root or stem to provide crops year after year. For example: cassava (Manihot ultissima) has a thick edible root, tapioca is also made from this plant; irish potato (Solanum tuberosum) are a modern tuber crop that is easily propagated by “eyes” (buds) on tubers; in Asia taro (Colocasia sp.) & West Indies, tannia (Xanthosma sp.) are both known to be early root crops, both have starch rich corms (swollen underground stems) and are easily propagated by buds on the corm. Later Developments in Agriculture The Romans were using crop rotation, manure, grafting, and experimenting with plant varieties by 200 CE. The Aztecs developed sophisticated irrigation system (=Chinampas) to expand areas of cultivation and yield (1100’s-1300’s). The high productivity of the region was a major reason why the Aztecs were able to dominate such a large region in such a short period of time. By the time the Spanish conquistadors arrived at Mexico City in 1519, the Aztec emperor was receiving annual offerings of: 7000 tons of corn, 5000 tons of chilies, 4000 tons of beans, 3000 tons of cocoa, 2 million cotton cloaks, several tons of gold, amber, and other valuables. Increased population growth allowed the development of large cities, even in ancient times and the exchange of crop plants worldwide. Continued agricultural advances resulted from selecting more productive strains of crop plants Industrialization lead to large scale agriculture. It greatly increased areas under cultivation and greatly increased productivity/acre. Today, in industrialized countries, all the food is produced by only 5% of

the population. Over 90% of all human caloric intake is provided by commercially grown plants. Of the more than 250,000 known species of plants, only a few species are exploited to any degree with the food plants that were 1st domesticated remaining as our primary staple crops today. Only 6 crops: wheat, rice, corn, potatoes, yams, &cassava provide ~80% of the total caloric intake for the world's population. Tomatoes and coffee are the only two major commercial plant crops that have been developed in the past 2000 years.

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Course Code: 19BTP205B Batch: 2019

Unit III
Improvement of crop plants

Unit III
Syllabus

Increase of Iron, Protein and Amino acid, golden rice, colours- Anthocyanins, betalains, crocin and crocetins. Flavours- capsaicin, vanillin, stevoside thaumatin. Developing vaccine and plantibodies, terminator technology and male sterility.

Iron is an essential micronutrient for almost all living organisms because of it plays critical role in metabolic processes such as DNA synthesis, respiration, and photosynthesis. Further, many metabolic pathways are activated by iron, and it is a prosthetic group constituent of many enzymes. An imbalance between the solubility of iron in soil and the demand for iron by the plant are the primary causes of iron chlorosis. Although abundant in most well-aerated soils, the biological activity of iron is low because it primarily forms highly insoluble ferric compounds at neutral pH levels. Iron plays a significant role in various physiological and biochemical pathways in plants. It serves as a component of many vital enzymes such as cytochromes of the electron transport chain, and it is thus required for a wide range of biological functions. In plants, iron is involved in the synthesis of chlorophyll, and it is essential for the maintenance of chloroplast structure and function. There are seven transgenic approaches and combinations, which can be used to increase the concentration of iron in rice seeds.
The first approach involves enhancing iron accumulation in rice seeds by expressing the ferritin gene under the control of endosperm-specific promoters.

The second approach is to increase iron concentrations in rice through overexpression of the nicotianamine synthase gene (NAS). Nicotianamine, which is a chelator of metal cations, such as Iron and zinc is biosynthesized from methionine via S-adenosyl methionine synthase.

The third approach is to increase iron concentrations in rice and to enhance iron influx to seeds by expressing the Fe^{+2} - nicotianamine transporter gene OsYSL2.

The fourth approach to iron biofortification involves enhancing iron uptake and translocation by introducing genes responsible for biosynthesis of mugineic acid family phytosiderophores (MAs).

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The fifth approach to enhance iron uptake from soil is the over expression of the OsIRT1 or OsYSL15 iron transporter genes.

The sixth approach to enhanced iron uptake and translocation is overexpression of the iron homeostasis-related transcription factor OsIRO2. OsIRO2 is responsible for the regulation of key genes involved in MAs-related iron uptake.

The seventh approach to enhanced iron translocation from flag leaves to seeds utilizes the knockdown of the vacuolar iron transporter gene OsVIT1 or OsVIT2. The present review discusses iron toxicity in plants with regard to plant growth and metabolism, metal interaction, iron-acquisition mechanisms, biofortification of iron, plant-iron homeostasis, gene function in crop improvement, and micronutrient interactions.

Effects of Iron on Plant Growth

Iron is the third most limiting nutrient for plant growth and metabolism, primarily due to the low solubility of the oxidized ferric form in aerobic environments (Zuo and Zhang, 2011; Samaranayke et al., 2012). Iron deficiency is a common nutritional disorder in many crop plants, resulting in poor yields and reduced nutritional quality. In plants, iron is involved in chlorophyll synthesis, and it is essential for the maintenance of chloroplast structure and function. Being the fourth most abundant element in the lithosphere, iron is generally present at high quantities in soils; however, its bioavailability in aerobic and neutral pH environments is limited. In aerobic soils, iron is predominantly found in the Fe^{+3} form, mainly as a constituent of oxyhydroxide polymers with extremely low solubility. In most cases, this form does not sufficiently meet plant needs. The visual symptoms of inadequate iron nutrition in higher plants are interveinal chlorosis of young leaves and stunted root growth. In waterlogged soils, the concentration of soluble iron may increase by several orders of magnitude because of low redox potential (Schmidt, 1993). Under such conditions, iron may be taken up in excessive quantities. However, it is potentially toxic and can promote the formation of reactive oxygen-based radicals, which are able to damage vital cellular constituents (e.g., membranes) by lipid peroxidation. Bronzing (coalesced tissue necrosis), acidity, and/or blackening of the roots are symptoms of plants exposed to above-optimal iron levels (Laan et al., 1991). Iron predominantly exists as Fe^{+3} chelate forms in the soil, and plants ultimately cannot absorb it under various physiological conditions such as high soil pH in alkaline soils. Thus, plants growing in high-pH soils are not very efficient at developing and stabilizing chlorophyll,

resulting in the yellowing of leaves, poor growth, and reduced yield. However, plants have developed sophisticated mechanisms to take up small amounts of soluble iron. Non-graminaceous plants release protons, secrete phenolics, reduce Fe^{+3}, and take up iron (Römheld and Marschner, 1983; Marschner, 1995a; Jeong and Guerinot, 2009; Cesco et al., 2010). Once iron is solubilized, Fe^{+3} is reduced to Fe^{+3} by a membrane-bound Fe reductase oxidase (Jeong and Connolly, 2009). Fe is then transported into the root by an iron-regulated transporter (IRT1). Ishimaru et al. (2011) reported that graminaceous plants rely on an Fe^{+3} chelation system during the secretion of mugineic acid (MA) family phytosiderophores. MAs are secreted to the rhizosphere through TOM1, and they then chelate Fe^{+3}. In rice, the resulting Fe-MA complex is transported by the yellow stripe family transporters (OsYSL15) (Nozoye et al., 2011). Rice plants also have the ability to take up the iron transporter (Ishimaru et al., 2006). Rout et al. (2014) screened 51 varieties of upland and lowland rice using different levels of iron (0, 50, 100, and 200 mM) in nutrient solution to study the toxicity effect. Out of 51 varieties, 16 varieties were tolerant (>200 mM Fe), 11 exhibited medium tolerance (<200 mM Fe), and 24 varieties were susceptible (<100 mM) to selected iron concentrations. Total chlorophyll, total proline, total phenol, total protein, and total carbohydrate content showed variation in both tolerant and susceptible varieties. The oxidative enzymes also showed variation among the tolerant and non-tolerant genotypes.

Typically, approximately 80% of iron is found in photosynthetic cells where it is essential for the biosynthesis of cytochromes and other heme molecules, including chlorophyll, the electron transport system, and the construction of Fe-S clusters (Briat et al., 2007; Hansch and Mendel, 2009). In the photosynthetic apparatus, two or three iron atoms are found in molecules directly related to photosystem II (PS-II), 12 atoms in photosystem I (PS-I), five in the cytochrome complex, and two in the ferredoxin molecule (Varotto et al., 2002). Such distributions show that iron is directly involved in the photosynthetic activity of plants and, consequently, their productivity (Briat et al., 2007). Iron availability is assumed to affect the natural distribution of species, and it may limit the growth of fast-growing economically important plants (Chen and Barak, 1982). Iron can also be potentially toxic at high concentrations. The ability of iron to donate and accept electrons means that if iron is free within the cell, it can catalyze the conversion of hydrogen peroxide into free radicals. Free radicals can cause damage to a wide variety of cellular structures, and they can ultimately

kill the cell (Crichton et al., 2002). To prevent that kind of damage, life forms have evolved a biochemical protection mechanism by binding iron atoms to proteins.

Protein and Amino acid enhancement in Major crop plants

Breeding for plant compositional traits to enhance nutritional quality or meet an industrial need are major plant breeding goals. High protein crop varieties (e.g., high lysine or quality protein maize) have been produced for use in various parts of the world. Different kinds of wheat are needed for different kinds of products (e.g., bread, pasta, cookies, semolina). Breeders have identified the quality traits associated with these uses and have produced cultivars with enhanced expression of these traits. Genetic engineering technology has been used to produce high oleic sunflower for industrial use; it is also being used to enhance the nutritional value of crops (e.g., pro-vitamin A golden rice). The shelf life of fruits (e.g., tomato) has been extended through the use of genetic engineering techniques to reduce the expression of compounds associated with fruit deterioration.

Breeding for high protein content in crop plants is perhaps the highest priority in improving the nutritional quality of plants because about 70% of the protein supply of human consumption is of plant origin. Further, cereals are deficient in some essential aminoacids and low in total protein. Maize was one of the first crops on which formal nutritional augmentation work was done. In 1896, C.G. Hopkins initiated a project to breed for high protein and oil content at the Illinois Agricultural Experimental Station. Work by T.B. Osborne in the early 1900s resulted in the fractionation and classification of proteins according to solubility properties. He and his colleague discovered zein (the prolamin or alcohol soluble fraction) as comprising the bulk of protein of maize endosperm. Later work in the mid 1900s by K.J. Frey demonstrated that breeding for protein augmentation primarily increased the zein content. There was a need to find a way to enhance the useful part of the protein. E.J. Mertz in 1964 discussed the nutritional effects of the opaque-2 gene in maize. The mutant gene increased the lysine content, called high lysine. High lysine research has since been conducted in sorghum. Another cereal food of world importance is rice. However, it has significant nutritional problems, being low in protein as well as completely lacking vitamin A. Rice nutritional augmentation was initiated in 1966 at IRRI (the International Rice Research Institute) in the Philippines. The vitamin A deficiency is being addressed using genetic engineering.

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Breeding for improved protein content

The key components of food that impact nutrition are carbohydrates, fats, proteins, minerals, water, vitamins, and fiber. The first three components provide calorific energy, while proteins, minerals and water play a role in the body tissue and structure. The roles of regulation and utilization are played by proteins, minerals, water, vitamins, and fiber. After satisfying calorific energy needs, proteins are the next most important nutritional component of a diet. Twentytwo amino acids are generally recognized in human nutrition, of which eight are essential for monogastric animals. The utilization efficiency of the entire protein is diminished if the diet is deficient in any of the essential amino acids.

Breeding high lysine content grain

Breeders using conventional methods of ear-to-row selection were able to increase total protein content of corn kernel from 10.9 to 26.6%. Unfortunately, because the protein of corn is about 80% zein and hence nutritionally inadequate, the high increase in total protein was nutritionally unprofitable to nonruminant animals. The zein fraction of the total protein is deficient in lysine and tryptophan. This deficiency was corrected in 1964 when researchers at Purdue University discovered mutant genes, called opaque-2 and floury-2, which increased the lysine content of the kernel. The patterns of expression of the mutant genes differ slightly. The opaque2 gene has a recessive gene action, whereas the “floury-2” gene exhibits a dosage effect. The resulting corn is called high lysine corn and has a characteristic soft and starchy endosperm. Consequently, the softer endosperm predisposes high lysine kernels to breakage, cracking, and rot. Generally, high lysine cultivars yield lower than their conventional counterparts. Cross-pollination with normal dent corn reverses the soft endosperm to normal dent endosperm. High lysine corn production must be done in isolated fields. The opaque-2 recessive gene increased the lysine content of the kernel from about 0.26–0.30% to about 0.34–0.37%. High lysine has been transferred into sorghum.

Essential amino acids in animal/human nutrition

Isoleucine, Alanine, Serine, Leucine, Arginine, Tryosine, Lysine, Cysteine, Asparagine, Methoinine, Glutamic acid, Glutamine, Phenylalanine, Glycine, Cystine, Threonine, Histidine, Hydroxyglutamic acid, Tryptophan, Proline, Valine and Norleucine.

Quality protein maize (QPM)

Quality protein maize(QPM) may be described as an extension of the improvement of high lysine maize. It is a high lysine product because it uses the opaque-2 gene. However, it is unlike the traditional high lysine maize because it lacks all the undesirable attributes of high lysine products (i.e., low yields, chalky-looking grain, and susceptibility to diseases and

insects pests). It looks like regular maize but has about twice the levels of lysine and tryptophan. QPM was developed by two researchers, K.V. Vasal and E. Villegas over three decades. They used conventional breeding methods to incorporate modifier genes to eliminate the undesirable effects of the lysine gene. The two scientists were rewarded with the World Food Prize in 2001 for their efforts

Industry highlights QPM: enhancing protein nutrition in sub-Saharan Africa

Introduction Maize is a major staple in sub-Saharan Africa and also constitutes an important source of food for children in particular. For example, children in Ghana grow well during the first six months of life but, thereafter, when breast milk ceases to be sufficient to sustain their rapid growth, malnutrition becomes normal. This nutritional trend is explained by the pervasive use of a thin gruel porridge made from maize or millet as the first weaning food fed to children. Few mothers supplement such cereal diets with other sources of protein, such as beans, fish or milk, due to ignorance about proper nutrition, high cost or lack of time. The cereals alone do not provide a balanced diet because they are low in lysine and tryptophan, essential amino acids, which cannot be synthesized by monogastric animals including humans (National Research Council, 1988). Normal maize, for example, has approximately 10% protein but the full amount is not utilizable by monogastric animals because the protein is low in lysine and tryptophan. When children are fed normal maize without any better-balanced protein supplement, they become malnourished and develop the protein deficiency disease called Kwashiorkor. In 1963, Mertz and his coworkers at the University of Purdue discovered a recessive mutant maize gene, opaque-2, which resulted in grain protein with approximately twice the quantities of lysine and tryptophan, the two limiting amino acids in ordinary maize (Mertz et al., 1964). There was an immediate upsurge of worldwide interest to develop nutritionally improved maize varieties. However, it was soon discovered that the gene conferring the improved nutritional quality also resulted in several undesirable agronomic traits, including low grain yield potential, unacceptable chalky grain type, high moisture at harvest, and high susceptibility to insects and disease attacks (National Research Council, 1988). When farmers rejected the early “high lysine” hybrids quickly released to them, the research in opaque-2 or high lysine maize waned markedly worldwide. However, unrelenting research continued for some 30 years at the International Maize and Wheat Improvement Center (CIMMYT), Mexico, resulting in the development of maize germplasm combining better protein quality with desirable grain yield potential and agronomic characteristics similar to normal maize (Bjarnason, 1990). This new source of nutritionally improved maize was the result of the accumulation of modifier genes in a rather complex breeding technique supported by a strong laboratory for analysis of protein quality (Vasal et al., 1993). The new material had normal looking hard endosperm grain type and was

designated Quality Protein Maize (QPM). At the time QPM germplasm became available, there were still doubts about the usefulness of QPM for farmer production and since researchers – especially those in developing countries – continued to show little interest in QPM, in 1991 CIMMYT therefore closed its research on QPM. It was at this time, however, that the Ghana Grains Development Project (GGDP) within the Crops Research Institute, Kumasi, Ghana, assigned one breeder full-time to initiate a QPM development project for Ghana. The Government of Ghana, the Canadian International Development Agency (CIDA), and Sasakawa Global 2000 (SG2000) provided research funding. The main objectives of QPM research in Ghana was to develop high and stable yielding QPM varieties with comparable performance to their normal counterparts, to demonstrate their nutritional advantages, and to promote their production, marketing and utilization.

Breeding approaches

The QPM germplasm used to initiate QPM breeding in Ghana was collected from CIMMYT, Mexico, in 1991. The germplasm included open-pollinated experimental varieties and early generation inbred lines from CIMMYT Populations 62 (white flint) and 63 (white dent). The maize streak virus disease was a major problem in Ghana at the onset of the QPM program. Therefore, the QPM germplasm was converted to streak virus disease resistance by backcrossing the susceptible materials to resistant sources obtained from the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria. This was followed by growing the lines under artificial streak pressure and self-pollinating resistant lines to produce S1 lines. Half-sib (HS) and S1 recurrent improvement schemes were employed to develop the first QPM variety in Ghana. In the HS procedure, about 300 families were grown in an isolation block. Three rows were detasseled before anthesis to constitute female rows and these were grown in alternation with one male row (not detasseled), which served as the pollen source. Equal numbers of seed from each family were composited to plant the male rows. At harvest, HS families with desired agronomic traits were selected. These families were planted again as ear-to-row and selfed to produce S1 lines. Twenty seeds of selected S1 lines were sent for protein quality analysis (basically measuring tryptophan levels in total protein) at CIMMYT, Mexico. At the same time, light tables were used to select modified grains in the S1 lines. On the basis of visual selection of HS lines in the field, the result of the analysis of protein quality (tryptophan level) in the laboratory and grain modification under light tables, S1 lines were selected and recombined in isolation to constitute the next cycle of improvement. A streak resistant QPM variety named Obatanpa (literally “Good Nursing Mother”) was released in 1992. This was a white dent medium maturity (105 days) variety. We also initiated a hybrid development program. We developed several inbred lines through inbreeding by selfpollination, and conducted early generation testing and topcross evaluation.

Diallel cross evaluations were used to determine the combining abilities of advanced inbred lines. Several single and three-way hybrids were developed. Open-pollinated varieties and hybrids developed in the project were first tested at the research stations located in the major agro-ecological zones in Ghana. The Ghanaian QPM hybrids performed equal to or better than local check hybrids. Consequently, three of the hybrids were released in Ghana and one was released in South Africa for production in Southern African countries. QPM germplasm development was supported by biochemical analysis of the grain at CIMMYT, Mexico and Ghana.

QPM nutritional studies

Despite the progress obtained in QPM germplasm development in Ghana, widespread doubts persisted about the usefulness of QPM technology. We conducted several collaborative animal feeding studies on pigs, chickens and rats to ascertain the nutritional advantages of QPM when used as human or animal food. The collaborative institutions included the CRI, SG2000 and the Animal Science Department of the Kwame Nkrumah University of Science and Technology, and the Health and Nutrition Department of the Ministry of Health.

Processed food from normal maize and QPM

Kenkey is a popular local food in Ghana. It is made from fermented maize meal. We studied the effect of processing and cooking on the nutritional quality of Kenkey made from normal maize or QPM (Ahenkora et al., 1995). The Quality Protein Maize, Obatanpa, and the normal maize, Okomasa, were processed into Kenkey. Weaning rats were fed ad libitum on Kenkey-based diets, which served as the sole source of protein and amino acid for 28 days. Analysis of samples of the Kenkey revealed that processing and cooking raw grains into Kenkey reduced the lysine content by 13% and the tryptophan content by 22% (Ahenkora et al., 1995). However, Kenkey from QPM contained 51% more lysine and 63% more tryptophan than Kenkey from normal maize. The individual average gain by rats fed on QPM Kenkey diet was 37.2 g compared with 16.2 g for normal-maize Kenkey – a 2.3-fold difference. Rats fed a QPM diet had better feed conversion ratio and higher protein efficiency ratio (PER) values than their counterparts fed a normal maize Kenkey diet.

Agricultural technology/nutrition impact study:

A series of studies investigated the impact of QPM utilization on community-based agricultural technology interventions in the Ejura-Sekodumasi district, Ashanti Region, Ghana. The study was done through the Ministry of Health Nutrition Division with collaboration from other agricultural and health institutions in Ghana, particularly CRI,

Ministry of Food and Agriculture (MOFA), and SG2000. The results showed that QPM enhanced growth relative to normal maize when fed to children.

Breeding challenges related to QPM

A series of experiments were conducted to dispel some of the doubts, myths and fallacies concerning QPM (Twumasi-Afriyie et al., 1996). QPM produces a lower grain yield than normal maize counterparts. In Ghana, it was shown that QPM varieties could produce better than their normal counterparts. Lysine and tryptophan were "heat labile" and would be destroyed during processing, thus QPM will lose its nutritional advantage during processing into local dishes. We demonstrated that the nutritional advantage was maintained when QPM was processed into the most popular local dishes (Ahenkora et al. 1995). QPM is conferred by a recessive gene and thus will lose its nutritional advantage in farmers' production plots, which are normally planted on small areas. We conducted an experiment in which we surrounded a one-acre field of QPM with a yellow endosperm normal maize with the same maturity, and allowed the two to cross freely. Results from two years of data at several locations showed a maximum of 10% contamination by the normal maize. The contamination was most pronounced within the 12 m of the QPM field nearest the normal maize and was most serious at the southwestern sector of the field due to the prevailing wind. The nutritional quality of the bulked grain from the most contaminated lot was still not significantly different from the non-contaminated QPM (based on a rat feeding study). QPM will not store well at the farm level. From our study, when weevils were introduced into grains of normal or QPM grain, there was no difference in the extent of damage incurred. All samples were equally damaged in a short period. Moreover, it was detected that, in general, post-harvest handling was very poor in Ghana and that available improved technology, if followed, could enable farmers to store both NM and QPM with minimal problems.

Golden Rice in development

At the start of the 1990s two university scientists, Ingo Potrykus from the ETH institute in Zürich and Peter Beyer from the University of Freiburg, were well aware of the blindness caused by vitamin A deficiency. They undertook the initiative to develop rice that produces provitamin A in the grain. To this end the researchers gained support from the Rockefeller Foundation, the European Commission, national governments in Asia and finally from the Bill and Melinda Gates Foundation. Because no rice varieties exist that produce provitamin A in the grain, this trait couldn't be introduced via traditional breeding, such as was the case with the sweet potato. Adding two genes from the narcissus and one from the soil bacteria Erwinia uredovora to the DNA of rice enabled the production of provitamin A in rice grains.

More than one gene is needed because the production of provitamin A is a multistep process. For some of these steps no genes are active in white rice grains.

The first version of the rice rich in provitamin A produced just over 1.6 micrograms of provitamin A per gram of rice. This proved a significant scientific breakthrough, as for the first time the entire production pathway of a nutrient had been introduced into a plant. The presence of provitamin A in the rice grains was even visible. Just as carrots are colored orange by their vast quantity of provitamin A, the GM rice grains are also yellow-orange in color. As such the rice was dubbed 'Golden Rice'. However, the concentration of provitamin A in initial Golden Rice was possibly too low to be effective in normally consumed amounts of rice. People would have to consume too much rice each day in order to reach the recommended daily intake of vitamin A. The new Golden Rice produced up to 23 times more provitamin A compared to its initial counterpart, making it more useful in the fight against vitamin A deficiency. To ascertain whether this would indeed work in practice, the GM rice was tested in nutritional experiments involving children aged between six and eight years in China. This research showed that Golden Rice could provide just as much vitamin A as provitamin A capsules, and more than spinach. Based on this data it was calculated that a bowl of 100-150 grams of boiled Golden Rice (equivalent to 50 grams of dry rice) provides 60% of the recommended daily intake of vitamin A for children. Considering that people in the Philippines eat around 330 grams of boiled rice a day, Golden Rice would provide enough vitamin A among population groups whose diet consists primarily of rice.

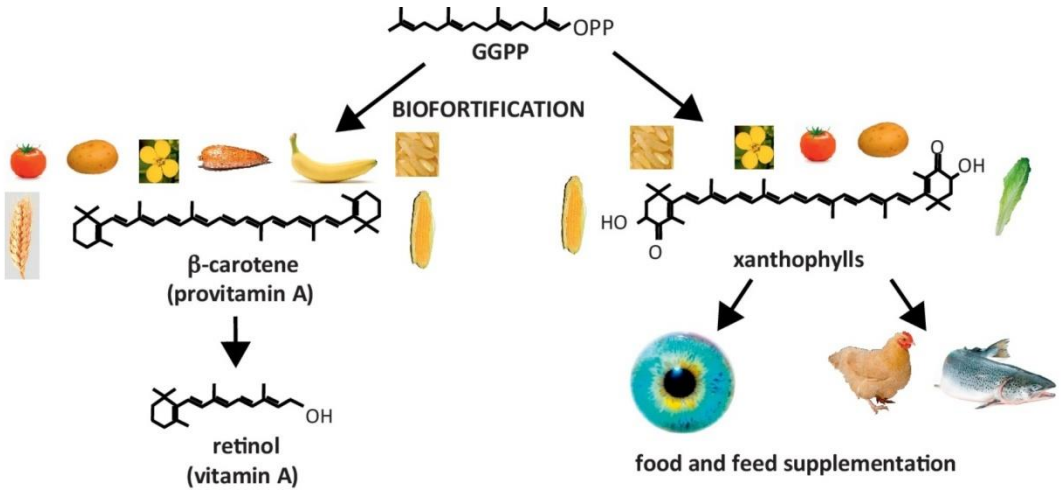
SAFFRON CROCUS (CROCUS SATIVUS L.)

Saffron is an expensive spice obtained from the dried styles of saffron crocus (Crocus sativus), whose distinctive flavor and color are due to picrocrocin, safranal and crocetin-derived apocarotenoids. The biosynthesis starts from the cleavage of zeaxanthin, to produce cyclic carotenoid VOCs (picrocrocin and safranal) and crocetin, which is eventually glycosylated to crocin (Figure 1). Some of the genes and enzymes involved in these steps have been studied. The Crocus CCDs so far characterized are similar to CCD1 and CCD4 enzymes, are differentially expressed in flower organs and CCD4s only contain predicted transit peptides for plastid (plastoglobule) localization (Rubio et al., 2008). The CCD1-like generic CsCCDs possess 9,10 (9',10') cleavage activity on various carotenoid substrates (Bouvier et al., 2003b; Rubio et al., 2008). CCD4- like CsCCD4a and CsCCD4b proteins (Rubio et al., 2008) are very similar (98-100% homology) to the CsZCD enzyme, previously reported to cleave zeaxanthin at the 7,8(7',8') positions and giving rise to crocetin dialdehyde (Bouvier et al., 2003b). CsCCD4 enzymes are longer than CsZCD and carry a plastid transit peptide. They perform a 9,10(9',10') cleavage, and are also able to cleave zeaxanthin, although the expected apocarotenoids could not be detected by neither LC nor GC (Rubio et al., 2008). Further investigation is needed to clarify the exact number, protein structure, and

enzymatic activity of Crocus CCD4/ZCD enzymes. Besides apocarotenoid volatiles, water-soluble crocetin glycosides are presumed to accumulate in vacuoles to confer pigmentation. A UDP-glucose crocetin 8-8'-glycosyltransferase enzyme was purified from cell suspensions and characterized (Côté et al., 2000), and the product of the stigma-expressed UGTCs2 gene was shown to glucosylate crocetin aglycones and glycosides in vitro (Rubio Moraga et al., 2004).

Carotenoids

Carotenoids are synthesized de novo by plants, where they play fundamental physiological roles as photosynthetic pigments and precursors for signaling molecules. They are also essential components of a healthy diet, as dietary antioxidants and vitamin A precursors. Vitamin A deficiency is a public health problem in developing countries, which has prompted a series of efforts toward the biofortification of plant-derived foods with provitamin A carotenoids (mainly b-carotene), giving rise to 'golden' crops. Since the 'golden rice' exploit, a number of biofortified crops have been generated, using transgenic approaches as well as conventional breeding. Bioavailability studies have demonstrated the efficacy of several 'golden' crops in maintaining vitamin A status. This review presents the state of the art and the areas that need further experimentation.



Preparation method of golden rice mate rich in crocin and geniposide

The invention discloses a preparation method of a rice mate rich in crocin and geniposide, particularly relates to a preparation method of a rice mate rich in crocin and geniposide by

utilization of a subcritical extraction technology, and belongs to the field of medicinal food and healthcare food. The preparation method includes three steps, namely, degreasing, desolventizing and cooking. More than 99.9% of liposoluble constituents in materials can be extracted by the subcritical extraction technology (with the oil content of dregs after the degreasing step being lower than 0.1%). The product can be directly used or be further processed as a golden rice mate to prepare jasmine golden rice, and the like. In addition, the biological activity of functional components in the extract is guaranteed and the product is

liable to store. Effective components which are the crocin and the geniposide are preserved in the dregs can be further widely applied. Comprehensive utilization of nutritions in the "dregs" after fat is extracted is guaranteed. The method has a wide application scope.

Crocin biosynthesis in *Crocus* has been proposed to proceed through a zeaxanthin cleavage pathway catalyzed by carotenoid cleavage dioxygenase 2 (CCD2), and followed by glucosylation reactions catalyzed by CsGT2 (UGT74AD1). In *Crocus ancyrensis* flowers, crocins with eight (crocin-1), seven (crocin-2), and six glucose (crocin-3) moieties accumulated both in stigma and tepals. We have characterized the structure of these highly glucosylated crocins and follow up their accumulation by high-resolution liquid chromatography coupled with diode array detector along the development of both tissues, and coupled to the isolation and analysis of the expression of eighteen genes (*PSY-I*, *PSY-II*, *PDS-(I-V)*, *ISO-ZDS*, *ZDS*, *CtrISO*, *LYC-I* and *II*, *BCH*, *CaCCD2*, *UGT74AD2*-5) related with the apocarotenoid metabolism in *C. ancyrensis* tepals and stigmas. Structure elucidation of crocin-1 and crocin-2 was done by the combined use of 1D and 2D [^{1}H, ^{1}H] (gCOSY and TOCSY and ROESY) and [^{1}H-^{13}C] NMR experiments, revealing that for crocin-1 was all-*trans*-crocetin O-[β-D- Glucopyranosyl)-(1→4)-(β-D-glucopyranosyl)-(1→2)]-O-[β-D-glucopyranosyl-(1→6)]-β-D-glucopyranosyl diester, while crocin-2 showed an identical structure except for the absence of one glucose residue in one end of the molecule. Crocins accumulation was not synchronically regulated in stigma and tepals, although in both cases crocins accumulation parallels tissue development, decreasing at anthesis. The expression of the carotenogenic genes *PSY*, *ZDS-V*, *BCH*, and *LCY-II* was correlated with crocins accumulation. In addition, *CaCCD2* and only one of the four glucosyltransferase encoding genes, *UGT74AD2*, were highly expressed, and the expression was correlated with high levels of crocins accumulation in stigma and tepals.

Apocarotenoids are derived from the oxidative cleavage of carotenoids and constitute a growing class of secondary metabolites with important functions in animals, insects, microorganism, and plants (Britton, 2008). In higher plants, apocarotenoids act as phytohormones signaling molecules and provide color to flowers and fruits (Auldridge et al., 2006; Walter et al., 2010). Among these compounds, there are two economically important

colored apocarotenoids produced by plants, crocetin, and bixin, used in the agro-food and in the pharmaceutical industry. Besides its color potential, crocetin has interesting biological properties that have been intensively studied with respect to its capacity in alleviating different diseases in humans (Hosseinzadeh and Nassiri-Asl, 2013). These health-promoting properties, along with its ability to act as natural colorant, have pushed an intense biotechnological interest to determine the biosynthetic pathways of crocetin in order to develop new, renewable sources producing these apocarotenoids (Winterhalter and Straubinger, 2000; Ahrazem et al., 2015).

The transcriptional regulation of carotenoid and crocetin pathways has been well documented in saffron stigmas (Castillo et al., 2005; Rubio et al., 2008; Moraga et al., 2009; Ahrazem et al., 2010). Biosynthetically, crocetin derives from zeaxanthin (Figure 1), synthesized in the chromoplast, using isopentenyl diphosphate (C5) derived from the methylerythritol-4-phosphate pathway (Pfander and Schurtenberger, 1982; Moraga et al., 2009). The conversion of two geranylgeranyl diphosphate (C20) molecules into phytoene (C40) represents the first committed step in the carotenoid pathway and is catalyzed by the enzyme phytoene synthase (Figure 1), (Cunningham and Gantt, 1998). Phytoene undergo a sequential series of desaturations and isomerizations to form all-*trans* lycopene. In plants, the desaturation and isomerization of phytoene to lycopene requires four proteins, a phytoene desaturase synthase and ζ-carotene desaturase, and two isomerases acting on ζ-carotene and poly *cis*-lycopene (Isaacson et al., 2002; Sandmann, 2009). Depending on the action and specificity of the cyclase enzymes, lycopene can then undergo cyclization to form β- or *ε*-ionone rings, yielding β-carotene and/or α-carotene. These cyclic carotenoids can be further modified by hydroxylation and epoxidation reactions (Nisar et al., 2015). β-carotene hydroxylation in both rings generates zeaxanthin, the precursor of crocetin (Figure 1). Zeaxanthin cleavage at the 7,8;7′,8′ double bonds by the carotenoid cleavage dioxygenase 2 (CCD2; Frusciante et al., 2014) generates crocetindial and two molecules of 3-hydroxy-β-cyclocitral. Both apocarotenoids are substrate of glucosyltransferases that transform them in crocins and picrocrocin, respectively (Moraga et al., 2004; Nagatoshi et al., 2012). Several crocins have been identified in saffron as crocetin mono (β-D-glucosyl) ester, crocetin di (β-D-glucosyl) ester, crocetin mono (β-gentiobiosyl) ester and crocetin (β-D-glucosyl) (β-gentiobiosyl) ester and crocetin (β-gentiobiosyl) (β-neapolitanosyl) ester (Pfister et al., 1996; Moraga et al., 2009).

Anthocyanins and betalaines

Anthocyanins usually appear red in leaf cells, but depending on their chemical nature and concentration, the vacuolar pH, and interactions with other pigments, they can

result in pink, purple, blue, orange, brown, and even black leaf colours (Schwinn and Davies 2004; Andersen and Jordheim 2006; Hatier and Gould 2007). Many of the published articles on plant defensive colouration have assumed red foliage to be the outcome of the production of anthocyanins, this despite the fact that other pigments – carotenoids, apocarotenoids, betalains, condensed tannins, quinones and phytomelanins – can also contribute to plant vermilion (Davies 2004). There is, moreover, a dearth of systematic information on the full complement of pigments in all plant organs at all developmental stages. This lack of data precludes detailed taxon-wide comparisons of the involvement of anthocyanin, or indeed any pigment, in plant defence. Clearly, if only visible cues (hue, lightness, and colour saturation) are involved in defence, the chemical nature of a pigment would be unimportant to a herbivore; red warnings would be similarly effective irrespective of whether they were generated by anthocyanins, carotenoids, or betalains. If, on the other hand, the efficacy of the warning relied on a combination of attributes, for example the reflection of red light plus the presence of a toxic or olfactory phenolic derived from an offshoot in the anthocyanin biosynthetic pathway, then the pigment type could be critical.

Anthocyanins are found in the cell vacuole, mostly in flowers and fruits, but also in leaves, stems, and roots. In these parts, they are found predominantly in outer cell layers such as the epidermis and peripheral mesophyll cells.

Most frequently occurring in nature are the glycosides of cyanidin, delphinidin, malvidin, pelargonidin, peonidin, and petunidin. Roughly 2% of all hydrocarbons fixed in photosynthesis are converted into flavonoids and their derivatives, such as the anthocyanins. Not all land plants contain anthocyanin; in the Caryophyllales (including cactus, beets, and amaranth), they are replaced by betalains. Anthocyanins and betalains have never been found in the same plant.

Sometimes bred purposely for high anthocyanin quantities, ornamental plants such as sweet peppers may have unusual culinary and aesthetic appeal.

Biosynthesis[edit]

Anthocyanins and carotenoids contribute distinctive pigmentation to blood oranges

1. Anthocyanin pigments are assembled like all other flavonoids from two different streams of chemical raw materials in the cell:
   - One stream involves the shikimate pathway to produce the amino acid phenylalanine, *(see phenylpropanoids)*

- The other stream produces three molecules of malonyl-CoA, a C_3 unit from a C_2 unit (acetyl-CoA),[56]

2. These streams meet and are coupled together by the enzyme chalcone synthase, which forms an intermediate chalcone-like compound via a polyketide folding mechanism that is commonly found in plants,
3. The chalcone is subsequently isomerized by the enzyme chalcone isomerase to the prototype pigment naringenin,
4. Naringenin is subsequently oxidized by enzymes such as flavanone hydroxylase, flavonoid 3′-hydroxylase, and flavonoid 3′,5′-hydroxylase,
5. These oxidation products are further reduced by the enzyme dihydroflavonol 4-reductase to the corresponding colorless leucoanthocyanidins,[57]
6. Leucoanthocyanidins once were believed to be the immediate precursors of the next enzyme, a dioxygenase referred to as anthocyanidin synthase, or, leucoanthocyanidin dioxygenase; flavan-3-ols, the products of leucoanthocyanidin reductase (LAR), recently have been shown to be their true substrates,
7. The resulting unstable anthocyanidins are further coupled to sugar molecules by enzymes such as UDP-3-*O*-glucosyltransferase,[58] to yield the final relatively-stable anthocyanins.

Thus, more than five enzymes are required to synthesize these pigments, each working in concert. Even a minor disruption in any of the mechanisms of these enzymes by either genetic or environmental factors, would halt anthocyanin production. While the biological burden of producing anthocyanins is relatively high, plants benefit significantly from the environmental adaptation, disease tolerance, and pest tolerance provided by anthocyanins.

In anthocyanin biosynthetic pathway, L-phenylalanine is converted to naringenin by phenylalanine ammonialyase (PAL), cinnamate 4-hydroxylase (C4H), 4-coumarate CoA ligase (4CL), chalcone synthase (CHS), and chalcone isomerase (CHI). Then, the next pathway is catalyzed, resulting in the formation of complex aglycone and anthocyanin through composition by flavanone 3-hydroxylase (F3H), flavonoid 3′-hydroxylase (F3′H), dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS), UDP-glucoside: flavonoid glucosyltransferase (UFGT), and methyl transferase (MT). Among those, UFGT is divided into UF3GT and UF5GT, which are responsible for the glucosylation of anthocyanin to produce stable molecules.[59]

In *Arabidopsis thaliana*, two glycosyltransferases, UGT79B1 and UGT84A2, are involved in the anthocyanin biosynthetic pathway. The UGT79B1 protein converts cyanidin 3-*O*-glucoside to cyanidin 3-*O*-xylosyl(1→2)glucoside. UGT84A2 encodes sinapic acid: UDP-glucosyltransferase.

Genetic analysis

The phenolic metabolic pathways and enzymes may be studied by mean of transgenesis of genes. The *Arabidopsis* regulatory gene in the production of anthocyanin pigment 1 (*AtPAP1*) may be expressed in other plant species.

Betalins

Betalains are a class of red and yellow indole-derived pigments found in plants of the Caryophyllales, where they replace anthocyanin pigments. Betalains also occur in some higher order fungi.[1] They are most often noticeable in the petals of flowers, but may color

the fruits, leaves, stems, and roots of plants that contain them. They include pigments such as those found in beets.

The name "betalain" comes from the Latin name of the common beet (*Beta vulgaris*), from which betalains were first extracted. The deep red color of beets, bougainvillea, amaranth, and many cacti results from the presence of betalain pigments.[2] The particular shades of red to purple are distinctive and unlike that of anthocyanin pigments found in most plants.

There are two categories of betalains:[3]

- **Betacyanins** include the reddish to violet betalain pigments. Among the betacyanins present in plants include betanin, isobetanin, probetanin, and neobetanin.
- **Betaxanthins** are those betalain pigments which appear yellow to orange. Among the betaxanthins present in plants include vulgaxanthin, miraxanthin, portulaxanthin, and indicaxanthin.

Plant physiologists are uncertain of the function that betalains serve in those plants which possess them, but there is some preliminary evidence that they may have fungicidal properties.[4] Furthermore, betalains have been found in fluorescent flowers.[5]

It was once thought that betalains were related to anthocyanins, the reddish pigments found in most plants. Both betalains and anthocyanins are water-soluble pigments found in the vacuoles of plant cells. However, betalains are structurally and chemically unlike anthocyanins and the two have never been found in the same plant together.[6][7] For example, betalains contain nitrogen whereas anthocyanins do not.[2]

It is now known that betalains are aromatic indole derivatives synthesized from tyrosine. They are not related chemically to the anthocyanins and are not even flavonoids.[8] Each betalain is a glycoside, and consists of a sugar and a colored portion. Their synthesis is promoted by light.[3]

The most heavily studied betalain is betanin, also called beetroot red after the fact that it may be extracted from red beet roots. Betanin is a glucoside, and hydrolyzes into the sugar glucose and betanidin.[2] It is used as a food coloring agent, and the color is sensitive to pH. Other betalains known to occur in beets are isobetanin, probetanin, and neobetanin. The color and antioxidant capacity of betanin and indicaxanthin (betaxanthin derived of l-proline) are affected by dielectric microwave heating.[9] Addition of TFE (2,2,2-trifluoroethanol) is reported to improve the hydrolytic stability of some betalains in aqueous solution.[10] Furthermore, a betanin-europium(III) complex has been used to detect calcium dipicolinate in bacterial spores, including *Bacillus anthracis* and *B. cereus*.[11]

Other important betacyanins are amaranthine and isoamaranthine, isolated from species of *Amaranthus*.

Betalain pigments occur only in the Caryophyllales and some Basidiomycota (mushrooms),[12] for instance Hygrophoraceae (waxcaps).[13] Where they occur in plants, they sometimes coexist with anthoxanthins (yellow to orange flavonoids), but never occur in plant species with anthocyanins.[14]

Among the flowering plant order Caryophyllales, most members produce betalains and lack anthocyanins. Of all the families in the Caryophyllales, only the Caryophyllaceae (carnation family) and Molluginaceae produce anthocyanins instead of betalains.[12] The limited distribution of betalains among plants is a synapomorphy for the Caryophyllales, though their production has been lost in two families.

Flavours- capsaicin

Chilli (Capsicum spp.) is an important commercial spice and vegetable crop for small and marginal farmers in Asia, Africa and South America and it is grown throughout the world. The name is derived from Capsicum' = Greek word 'kapto', meaning "to bite" or "to swallow." Almost 400 types of chillies are grown throughout the world. Among the 5 cultivated species of the genus Capsicum, C. annuum is the most widely cultivated in India for its pungent (chilli syn. hot pepper) and non-pungent (sweet pepper syn. capsicum, bell pepper) fruits. The cultivation of C. frutescens, C. chinense, and C. baccatum is limited and usually restricted to homestead gardening in different regions. It comprises numerous chemicals including steam volatile oils, fatty oils, capsaicinoids, carotenoids, vitamins, proteins, fibres and mineral elements (Bosland and Votava, 2000) [7].

Importance An important part of daily diet.• Key Element in many regional cuisines, pickles, soups, sauce, Salads, curries etc. due to• its unique flavor, aroma and colour. Increase the taste and palatability.• Fresh green capsicum contains more vitamin C than citrus fruits and fresh red chilli has• more vitamin A than carrot (Than et al. 2008). Chillies are low in sodium and cholesterol free.• Medicinal Properties are found Stimulate blood circulation• improves the digestion process• rich source of antioxidants• source of natural bactericidal agents• Apart from medicinal uses, chilli also used in cosmetic,• liquor industries and as a weapon for self -defense (chilli spray).

Capsicum annuum, bell, sweet, or chilli pepper—with cultivated varieties including bell, sweet, chilli, and paprika peppers—is a perennial herbaceous plants in the Solanaceae (nightshade family), which originated in Central and South America and the Caribbean and was domesticated over 5,000 years ago. Peppers from C. annuum have been developed into numerous varieties that are now cultivated around the world for sweet and hot varieties of green and red bell peppers and chilli peppers, that are one of the world's most widely used spices, with dried forms including paprika, chili powder, and cayenne. Capsaicin, which is obtained from C. annuum and other Capsicum species, is an intense skin and eye irritant, and is the ingredient used in pepper sprays sold for selfdefense. However, it also has numerous medical uses, including topical pain relief for muscle soreness, shingles, skin irritations, and rheumatism, and also as an antiinflammatory. Recent medical research has also documented antimicrobial and antifungal activity of capsaicin obtained from several Capsicum species, and on-going studies are exploring its use in cancer treatment. Capsicum chinense Capsicum chinense is a species of chilli pepper native to the America. C. chinense varieties are well known for their exceptional heat and unique flavors. Some taxonomists consider them to be part of the species C. annuum, and they are a member of the C. annuum complex. C. annuum and C. chinense can generally be identified by the number of flowers or fruit per node, however one for C. annuum and two to five for C. chinense, though this method is not always correct. The two species can also hybridize and generate inter-specific hybrids. It is believed that C. fruitescens is the ancestor to the C. chinense species Resistance to TSWV was found in several C. chinense accessions, including PI152225 and PI159236 (Kenyon et al. 2014) [14] . The resistance is controlled by the dominant gene Tsw and is expressed as a hypersensitive-like localization of the virus (Boiteux & de Ávila, 1994) [5] . This gene has been fine mapped to a 259-kb region of the distal portion of chromosome P10 where 22 genes are predicted, among which eight show annotations of NBS-LRR resistance proteins (Hoang et al., 2013) [16, 37] . This region is closely linked to, or may contain, the dominant potyvirus resistance genes Pvr4 and Pvr7. The resistance conferred by Tsw is broken at high temperatures; it depends on plant age, with young plants being more susceptible.

Sources of biotic stress resistance in Chillies Development of biotic resistant cultivars has been a part of the plant breeder's tool since long time. Cultivation of resistant or tolerant cultivars is one of the best options to minimize the losses due to disease/insect occurrence. This is especially at this juncture, when there is growing public sensitivity about the environmental pollution and residual effects on produce due to the indiscriminate use of hazardous chemicals and emergence of new races/biotypes. For the development of resistant varieties and pre-breed lines, sources of resistance are the prerequisite and backbone of breeding programme. Such sources may be present in the indigenous cultivars, land races, folk cultivars, semi-wild relatives and allied species of the vegetable crops. Chilli is highly susceptible to a number of fungal, bacterial and virus diseases (Rahim and Samraj, 1974) [50] . But the most serious are anthracnose (fruit rot and die back caused by Colletotrichum spp.), powdery mildew (Leucillula taurica), cercospora leaf spot (Cercospora capsici), bacterial leaf spot (Xanthomonas campestris Pv vesicatoria) and wet rot (Choanephora cucurbitarum). TMV, CMV, PVX, PVY, chilli mosaic virus and tobacco leaf curl virus are the most common viral disease in this crop (Singh and Kaur, 1990; Saini and Rathan, 1971) [52] . Yield losses ranged from 80 to 100% has been reported in case of early infection of viral diseases. Resistance sources in chillies crop have been reported for anthracnose, phytopthora blight, bacterial wilt, fruit rot and viral complexes. Sanjay et al., (2006) [53] screened 307 lines belonging to four cultivated and one wild species against pepper leaf curl virus under field and artificial conditions. Out of which only three genotypes, viz., GKC - 29, BS-35 and EC-497636 showed no symptoms. These lines should be used for future breeding programme against leaf curl viruses in combination with modern biotechnological tools. Important insects which attack chillies crop are thrips, mites, pod borer and aphids. These are more serious in tropical region of India and often causing 25 to 50% yield losses (Ananthakrishnan, 1973). The resistance source in chillies crop to various insects are summarized in table. (Tewari et al., 1985). Punjab Agricultural University, Ludhiana has developed 18 multiple disease resistant lines in chillies crop (Singh and Kaur, 1990). Among these, 'Punjab Lal' (S-118-2) has been released as multiple resistant chilli variety in 1985 for general cultivation in the State (Thakur et al., 1987). The other important multiple resistant lines are 'Perennial', 'BG-1', 'Loral', 'Tiwari', 'Indonesian Selection', etc. these are not only found resistant to various fungal and viral diseases in India but also in France, Hungary, Spain, Malaysia, Korea, USA and Taiwan. Another variety 'Pant C-1' found resistant to mosaic and leaf curl virus, has been released by Pant Nagar Agricultural University, Pant Nagar (U.P.) for general cultivation in the state. Indian Agricultural Research Institute has also released 'Pusa Sada Bahar' chilli variety resistant to CMV, TMV and leaf curl virus in 1989.

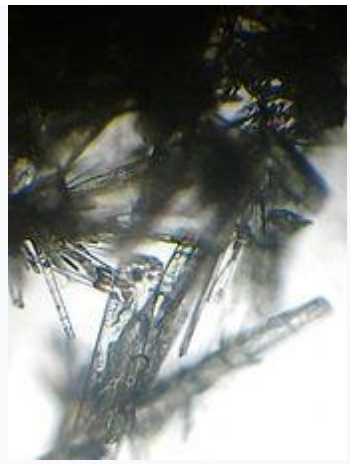
Vanillin

Vanillin is an organic compound with the molecular formula $C_8H_8O_3$. It is a phenolic aldehyde. Its functional groups include aldehyde, hydroxyl, and ether. It is the primary component of the extract of the vanilla bean. Synthetic vanillin is now used more often than natural vanilla extract as a flavoring agent in foods, beverages, and pharmaceuticals.

Vanillin and ethylvanillin are used by the food industry; ethylvanillin is more expensive, but has a stronger note. It differs from vanillin by having an ethoxy group ($-O-CH_2CH_3$) instead of a methoxy group ($-O-CH_3$).

Natural vanilla extract is a mixture of several hundred different compounds in addition to vanillin. Artificial vanilla flavoring is often a solution of pure vanillin, usually of synthetic origin. Because of the scarcity and expense of natural vanilla extract, synthetic preparation of its predominant component has long been of interest. The first commercial synthesis of

vanillin began with the more readily available natural compound eugenol (4-allyl-2-methoxyphenol). Today, artificial vanillin is made either from guaiacol or lignin.



Vanillin crystals extracted from vanilla extract

Lignin-based artificial vanilla flavoring is alleged to have a richer flavor profile than oil-based flavoring; the difference is due to the presence of acetovanillone, a minor component in the lignin-derived product that is not found in vanillin synthesized from guaiacol.

Synthetic vanillin became significantly more available in the 1930s, when production from clove oil was supplanted by production from the lignin-containing waste produced by the sulfite pulping process for preparing wood pulp for the paper industry. By 1981, a single pulp and paper mill in Thorold, Ontario supplied 60% of the world market for synthetic vanillin.[9] However, subsequent developments in the wood pulp industry have made its lignin wastes less attractive as a raw material for vanillin synthesis. Today, approximately 15% of the world's production of vanillin is still made from lignin wastes[10], while approximately 85% synthesized in a two-step process from the petrochemical precursors guaiacol and glyoxylic acid.[4][11]

Beginning in 2000, Rhodia began marketing biosynthetic vanillin prepared by the action of microorganisms on ferulic acid extracted from rice bran. At 700 USD/kg, this product, sold under the trademarked name Rhovanil Natural, is not cost-competitive with petrochemical vanillin, which sells for around 15 USD/kg.[12] However, unlike vanillin synthesized from lignin or guaiacol, it can be labeled as a natural flavoring.

Vanillin is most prominent as the principal flavor and aroma compound in vanilla. Cured vanilla pods contain about 2% by dry weight vanillin; on cured pods of high quality, relatively pure vanillin may be visible as a white dust or "frost" on the exterior of the pod.

It is also found in *Leptotes bicolor*, a species of orchid native to Paraguay and southern Brazil,[13] and the Southern Chinese red pine.

At lower concentrations, vanillin contributes to the flavor and aroma profiles of foodstuffs as diverse as olive oil,[14] butter,[15] raspberry,[16] and lychee[17] fruits.

Aging in oak barrels imparts vanillin to some wines, vinegar,[18] and spirits.[19]

In other foods, heat treatment generates vanillin from other chemicals. In this way, vanillin contributes to the flavor and aroma of coffee,[20][21] maple syrup,[22] and whole-grain products, including corn tortillas[23] and oatmeal.[24]

Thaumatin

Thaumatin is a mixture of intensely sweet proteins (thaumatin I and II), extracted with water from the arils of the fruit of *Thaumatococcus daniellii* together with minor amounts of plant constituents. It is an odourless, cream-coloured powder and functions primarily as a flavour enhancer and as a HIS. The taste characteristics are the slow onset of sweetness and a sweet aftertaste. It is soluble in water.

Thaumatin is nontoxic and makes an insignificant contribution to the normal protein intake. Therefore the WHO JEFCA in 1985 specified it to have no ADI. Thaumatin is about 2000 times sweeter than sucrose. Thaumatin is an approved sweetener in the EU for limited uses; currently there is no EU approval for bakery applications. It has a GRAS listing in the United States.

Thaumatin is a 22 kDa sweet protein that was isolated from the arils of the katemfe fruit of *Thaumatococcus daniellii* Benth, which is native to West Africa, by van der Wel and Loeve. It is a basic protein with an isoelectric point of approximately 12 and is 1600 times sweeter than sucrose. It also gives a cooling sensation and a slight licorice aftertaste. A water–sweet aftertaste was also reported.[57] Thaumatin is cultivated on a commercial scale and used as a sweetener, flavor enhancer, and flavor modifier. An aqueous solution of commercially available thaumatin is stable under the conditions of pH 2–10. There may be several related proteins in the plant, but there are two main forms: thaumatins I and II. Thaumatins I and II are each composed of 207 amino acids with eight intramolecular disulfide bonds shown in the figure. Thaumatins I and II differ in the amino acid sequence at 46, 63, 67, 76, and 113, which suggests that the two proteins are 98% identical.[58,59] Since the results of amino acid sequencing of the proteins were inconsistent with those of cDNA,[59] Lee *et al*. reinvestigated and isolated two proteins named thaumatins A and B, and found that they differ in only one amino acid at position 46, that is, Asn for A and Lys for B. The residue at 113 in thaumatin I is Asn, whereas it is Asp in thaumatins II, A, and B. Furthermore, thaumatin I did not make a refolding product, while thaumatins A and B expressed in yeast showed intense sweetness after refolding. Therefore, it has been suggested that there might have been an error in the determination of the residue at 113.[60] The tertiary structure of thaumatin I was analyzed by X-ray at resolutions of 3.1[61] and 1.65 Å.[62] It has been reported that thaumatin elicits a sweet taste in humans, and caused a significant electrophysiological response in the chorda tympani and glossopharyngeal nerves in the Old World monkey, but not the guinea pig or rat.[63] However, it was revealed that in Slc:ICR mice, chorda tympani and taste receptor cell response profiles and the behavioral results for monellin and thaumatin are similar to the response profiles for sucrose.[64] Thaumatin has been approved as a sweetener in Israel and Japan. In the United Nations, it is listed in Table III of

the Codex General Standard for Food Additives (GSFA), which means that it is permitted for use in food in general.

Plant-based vaccine:

Plant-based vaccine technologies involve the integration of the desired genes encoding the antigen protein for specific disease into the genome of plant tissues by various methods. *Agrobacterium*-mediated gene transfer and transformation via genetically modified plant virus are the common methods that have been used to produce effective vaccines. Nevertheless, with the advancement of science and technology, new approaches have been developed to increase the efficiency of former methods such as biolistic, electroporation, agroinfiltration, sonication, and polyethylene glycol treatment. Even though plant-based vaccines provide many benefits to the vaccine industry, there are still challenges that limit the rate of successful production of these third-generation vaccines. Even with all the limitations, continuous efforts are still ongoing in order to produce efficient vaccine for many human and animals related diseases owing to its great potentials. This paper reviews the existing conventional methods as well as the development efforts by researchers in order to improve the production of plant-based vaccines. Several challenges encountered during and after the production process were also discussed.

Agrobacterium is a Gram-negative soil pathogenic bacterium that naturally will infect the plants and transfer their genes (T-DNA) to the nucleus of the plant cells [17, 19]. Two strains of *Agrobacterium* species that have been commonly used as a biological vector are *Agrobacterium tumefaciens* (*A. tumefaciens*) and *Agrobacterium rhizogenes* (*A. rhizogenes*). The main difference between these two species is the plasmid that they carry. *A. tumefaciens* carries tumour-inducing plasmid (Ti-plasmid), while *A. rhizogenes* carries root-inducing plasmid (Ri-plasmid) [18, 37]. However, *A. tumefaciens* is the most preferred strain by researchers for stable expression of the desired protein. In the Ti-plasmid, there are genes encoding for plant hormones such as auxin and cytokinin synthesis, which will induce tumour tissue in plants. However, for vaccine production, these genes will be deleted to form disarmed Ti-plasmid and heterologous gene is inserted forming a recombinant plasmid vector [37]. The recombinant plasmid vector is transformed into *A. tumefaciens* and with the help from *vir* gene of the bacterium, the introduced heterologous gene is transferred by the transformed bacterium and integrated into the host plant nuclear genomic DNA by nonhomologous recombination at random sites [17, 19, 37]. The transformed bacteria are transferred into the plant leaves by soaking the leaves in the *A. tumefaciens* culture. This method is able to yield a stable integration of the transgene into the genome of the plant [33, 38].

Much research conducted prior to 2000 and early 2000 produced various vaccines through *Agrobacterium*-mediated transformation system in dicotyledonous plant models. For instance, Arakawa et al. successfully expressed gene encoding for cholera toxin B subunit

protein in potato leaf explants using *A. tumefaciens* [39]. Similarly, potato has been transformed to produce VP60 protein against rabbit hemorrhagic disease virus, in which rabbits immunized with the potato's leaf extract showed increased anti-VP60 antibody titers and were protected against the hemorrhagic disease [40]. These discoveries have led to the production of more diverse antigens in various crop species. *Helicobacter pylori* TonB protein was expressed in transgenic *Arabidopsis thaliana* through this method [41]. The antigen produced was recognizable by rabbit anti-TonB antiserum and suitable to be used as vaccine against *Helicobacter* infections by oral administration. In addition, Li et al. showed that hepatitis B surface antigen gene was able to be introduced into tomato plants mediated by *A. tumefaciens* [42]. *Agrobacterium*-mediated transformation has also been used for the production of antigens such as heat-labile enterotoxin from *Escherichia coli*, Norwalk virus capsid protein, hepatitis B surface antigen, and transgenic alfalfa expressing proteins from the foot and mouth disease virus by using potato model [43].

Plantibodies:

A plantibody is an antibody that is produced by plants that have been genetically engineered with animal DNA encoding a specific human antibody known to neutralize a particular pathogen or toxin. The transgenic plants produce antibodies that are similar to their human counterparts, and following purification, plantibodies can be administered therapeutically to acutely ill patients or prophylactically to at-risk individuals (such as healthcare workers). The term plantibody and the concept are trademarked by the company Biolex.

A plantibody is produced by insertion of genes encoding antibodies into a transgenic plant. The plantibodies are then modified by intrinsic plant mechanisms (N-glycosylation).[3] Plantibodies are purified from plant tissues by mechanical disruption and denaturation/removal of intrinsic plant proteins by treatment at high temperature and low pH, as antibodies tend to be stable under these conditions. Antibodies can further be purified away from other acid- and temperature- stable proteins by capture on commercially produced protein A resins. Production of antibodies in whole transgenic plants, such as species in the genus Nicotiana, is cheaper and safer than in cultured animal cells.[4]

Terminator Technology

The terminator technology is a genetically engineered suicide mechanism that can be triggered off by specific external stimuli. The preferred trigger is antibiotic tetracycline, which is applied to seeds. As a result of which the seeds of the next generation will self-destruct by auto-poisoning. The main version of the terminator includes a set of three novel genes inserted into one plant. However, there is another version, which divides two or three genes on to two plants that are later to be cross-pollinated. The ultimate outcome is a dead seed in the following generation. Many consider terminator technology a problem due to the

fact that the top 10 largest seed companies globally control half the world's commercial seed market. Therefore, if terminator technology is commercialized, corporations will most likely try to incorporate this technology into all of their seeds. This would secure a much stronger monopoly on the seed market compared to patents because this technology would ensure that it is impossible for farmers to re-use their once harvested seeds.

One of the biggest myths perpetuated by the advocates of modern biotechnology is that these technologies, and especially genetic engineering, are likely to provide a solution to world hunger. While technology brings relief to life‟s drudgery, it also carries social, economic and ecological costs. This side effect of technical development has become obvious with the advent of the green revolution, which has led to decrease in biodiversity and an increase in pesticides use. Biotechnology and genetic engineering are revealed as chemical free solutions to the problems created by the technology of the green revolution. The terminator gene technology, or genetic use restriction technology (GURT), is the genetic modification of plants to make them produce sterile seeds in second generation which is also famous as a "suicide seeds". It is a biotechnological innovation patented in the United States that present great danger to agriculture and food security worldwide, particularly in developing countries. Scientists have questioned the technology on ethical as well as scientific grounds. This technology was patented by U.S. Department of Agriculture and the seed com pany, Delta and Pine Land Company – a subsidiary of the seeds and agrochemical multinational Monsanto/American Home products. The technology has been appropriately named "Terminator" by the Canadian governmental organization, Rural Advancement Foundation International (RAFI), which has spearheaded an international campaign against it. Terminator alters the expression of certain genes in plants so that plants terminate their reproductive switch, about the embryo and make themselves sterile. Such plants then produce seed that cannot germinate. Monsanto described this technology as a "gene protection technology".

ADVANTAGES:

1. This technology will induce private sector to make more investment in research and development of pure line varieties and open pollinated varieties because in these varieties the farmers do not change the seeds each years.
2. Farmers will use new seeds every year leads to maximum production.
3. This will result in stiff competition between the public and private sector institutions and ultimately the farmers will benefit through this technology.

CONCLUSION:

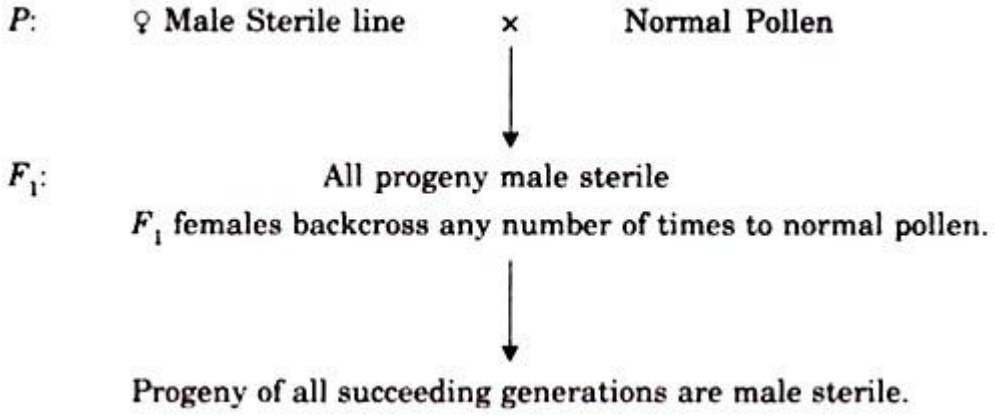
Food is a big business all around world and multinational companies would only be happier if farmers from all around the world are forced to come back to them, year after year, to buy seeds. Ina country like India, agriculture research if it is to be relevant and realistic must be in collaboration with farms and farmer organizations

and must be sensitive to the economic, social and conceptual framework within which farming communities work and make decisions. The terminator gene technology may be a good one for American agriculturists because there are only two percent of people in this field. Here in India we have more than 75 percent of our population who are engaged in agriculture. Therefore, what is relevant to them may not be relevant to us. Hence, the research should not be completely business oriented but it must be service oriented so that findings will reach to the farming community in India.

Male Sterility:

In plants, male sterility can be caused either by mitochondrial genes with coupled nuclear genes or by nuclear genes alone; the resulting conditions are known as cytoplasmic male sterility (CMS) and genic male sterility (GMS), respectively. CMS and GMS facilitate hybrid seed production for many crops and thus allow breeders to harness yield gains associated with hybrid vigor (heterosis). In CMS, layers of interaction between mitochondrial and nuclear genes control its male specificity, occurrence, and restoration of fertility. Environment-sensitive GMS (EGMS) mutants may involve epigenetic control by noncoding RNAs and can revert to fertility under different growth conditions, making them useful breeding materials in the hybrid seed industry. Here, we review recent research on CMS and EGMS systems in crops, summarize general models of male sterility and fertility restoration, and discuss the evolutionary significance of these reproductive systems.

The failure of pollen formation results in male sterile plants. In some crop plants male sterile mutants occur in which inheritance of male sterility follows one of the following two patterns: those in which the trait is inherited through a single recessive chromosomal gene segregating in Mendelian ratios; secondly, those that show maternal transmission. Rhoades in 1933 described maternal inheritance of male sterility in Zea mays.



In the cross shown above, the process of repeated backcrossing results in substitution of all the chromosomes of the fertile line for those of the male sterile line. The experiment therefore, not only shows cytoplasmic maternal transmission, but also confirms that chromosomal genes are not responsible for male sterility.

Iojap Strain of Maize:

The iojap strain of maize is characterised by green and white stripes on the leaf. The name iojap originates from two parental strains Iowa which is green and Japonica, a striped variety.

In crosses between iojap and green varieties when iojap is used as the male parent, the trait is inherited according to Mendelian pattern with F_1 progeny all green, and F_2 segregating into 3/4 green and 1/4 iojap. But in the reciprocal cross where iojap is used as the female parent, the F_1 plants showed all three phenotypes viz. green, white and striped.

P:	Iojap	×	Green	
	iijj	↓	IIjj	
F_1:		Iijj		
Phenotypes:	Green, White and Striped			
F_1	♀ Striped	Iijj	×	IIjj Green ♂
F_2	Genotypes:	Iijj		IIjj
	Phenotypes	Green, White, and Striped		Green, White, and Striped

It was found that the iojap gene in the homozygous recessive condition (ii) causes some of the plastids to mutate giving rise to colourless plastids. The mixture of green and colourless plastids accounts for the origin of striped plants. Once created, further transmission of the striped character takes place maternally through the egg cytoplasm as evident from the phenotypes of the F_2 progeny of the cross above.